# Cell Interactions and Vaccines of Tomorrow

Edited by R. V. PETROV

Advances in Science and Technology in the USSR

Biology Series

MIR Publishers
Moscow

# Cell Interactions and Vaccines of Tomorrow

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This book concentrates on the achievements of a group of Soviet researchers—immunologists, immunogeneticists, and chemists whose work has for decades concerned the most urgent problems of modern immunology. Special emphasis is given to original research by the authors of this volume. The discussion would be meaningless, of course, without substantiation from the current world literature. Nor can the contribution of a research team to a given body of knowledge be properly assessed without a firm understanding of the general principles governing the field. In addition to discussing their own experimental data, therefore, the authors analyse the most interesting works by Soviet and foreign researchers which provide a valuable illustrative material and reflect the rapid progress in immunology. Experiments conducted by the authors of the book during the last decade make up the core of this volume.

The chapter "Dictatorship of the Lymphocyte" deals with the ability of lymphocytes, the key cells of the immune system, to interact with stem cells, the main cells of haemopoiesis. This phenomenon was first discovered by the authors of the book.

Studies of the regulatory role of bone marrow in the immune response have led to a discovery of a new level of cell interactions related to mature antibody producers rather than to immunogenic precursors. At this level, the stimulating effects of bone marrow cells are expressed by a soluble mediator—a stimulator of antibody producers both *in vitro* and *in vivo*.

The chapter "Vaccines of Tomorrow" deals with a phenotypic correction of genetic control over immunity, a complicated, but, nevertheless, very exciting field of research that may lead to the development of entirely immunologic principles.

All the research presented here has one ultimate goal—to gain insight into function of each individual link in the immune system and to control its function directly.

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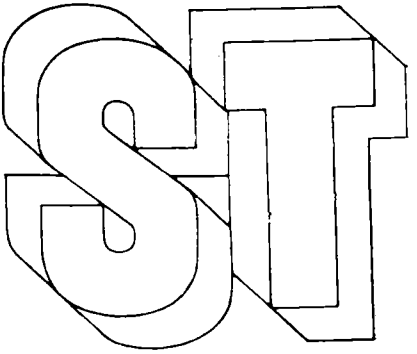

# Cell Interactions and Vaccines of Tomorrow

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Mir Publishers
Moscow

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КЛЕТОЧНЫЕ ВЗАИМОДЕЙСТВИЯ И ВАКЦИНЫ БУДУЩЕГО

Под редакцией академика АМН СССР Р. В. Петрова

First published 1984

*На английском языке*

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# Foreword

"Cell Interactions and Vaccines of Tomorrow", by a group of well-known Soviet authors, concerns one of the most urgent problems of modern public health care—the development of large-scale immunopreventive measures against various infectious diseases.

Bacterial, viral, and parasitogenic infections, long primary causes of morbidity and mortality in both developed and developing countries, are still dangerous to man. Up to 60 per cent of all fatalities are due to pathogenic infections, 70 per cent of all cases are caused by acute or chronic infections. During 1970s, the mortality rate in in-patient units amounted to 100,000 cases annually in the United States alone.

Public vaccinations have long been recognized as a highly effective means of immunoprophylaxis against acute infectious diseases of various etiology. The following facts support this view-point.

1. Significant achievements have been attained in the field of immunoprophylaxis against such important infectious diseases as diphtheria, measles, and polyomyelitis. Soviet public health services provide a clear example of the success of these programs.

2. Extensive vaccination has culminated in complete eradication of smallpox world-wide.

3. Bitter experience has demonstrated that the cessation of systematically implemented immunopreventive measures (even against a relatively favourable epidemiological background) inevitably leads to a sharp increase in the morbidity rate. Thus, in 1974, Japanese medical authorities prohibit further immunization of children against whooping-cough, because the cellular vaccine used at the time significantly aggravated clinical indices of the disease in a number of cases. As a result, the morbidity four years later amounted to 12,105 cases (compared to 393 in the vaccination period), of which 41 were fatal. Similar situations occurred recently in Great Britain and Sweden as well.

4. Even the most effective antibiotic therapy does not influence the circulation of a pathogen in a given community appreciably.

5. The number of environmental bacterial strains resistant to a very broad spectrum of antibiotics has considerably increased.

6. The correct implementation of immunopreventive measures has been significant economically. According to WHO statistics, full eradication of smallpox saves the mankind 1,000,000 US dollars every year.

In the last few decades, vaccines have been used widely as the most radical means in the global fight for human health and longevity.

At the Twenty-Seventh Session of the World Health Assembly, a resolution was passed, calling on WHO member-states to develop and further implement a program of immunization and epidemiological control over diphtheria, whooping-cough, tetanus, measles, tuberculosis, smallpox, and other infectious diseases. This project, commonly known as a comprehensive immunization program, is aimed at further reducing morbidity and mortality from infections to a reasonably low level. Special attention is given to developing countries, where 80,000,000 babies are born per year. In accordance with WHO prognoses, correct implementation of immunopreventive measures may lead to full eradi-

cation of measles and tetanus in newborns in the European region by the year of 2000.

Although the successes in controlling infectious diseases on a world-wide scale are indisputable, the selection of vaccines in current use is far from satisfactory. To this day, adequate immunological protective means against parasitogenic infections that affect 1,000 million people do not exist. WHO experts have calculated that the efficiency and, in some cases, safety of conventional vaccines are less than adequate, while the high cost of many new vaccines (e.g., vaccine against viral hepatitis B) is often a considerable obstacle to their large-scale application.

The latest achievements in the field of molecular biology, genetics, and immunology have opened new exciting prospects for the development of a new generation of inexpensive, highly effective vaccines with minimal side effects.

The search for new vaccines has developed along two main lines, biotechnological and immunological. The first approach is based on the development of entirely new ways of selection of antigenic material for creating vaccines. Such research employs gene engineering and chemical synthesis to obtain protein molecule fragments mimicking the antigenic structures of the parasite that triggers the immune response in the host. Despite optimistic prognoses, however, at its present level biotechnology cannot cope with all the problems arising from the synthesis of new vaccines, since the products of chemical and bacteria synthesis possess low activity and thus cannot afford adequate protection against the infection. This difficulty may be overcome with methods that amplify immune response to weak antigens.

This book includes original work by Soviet researchers devoted to the synthesis of a new type of immunopreventive drugs capable of evoking high immune response even in low-responder individuals. Such vaccines are an effective tool in the implementation of planned vaccination, especially in cases in which the spread of an infection on a given territory must be prevented.

The new vaccine synthesis theory advanced by a group of investigators from Moscow is aimed at solving another complicated problem of immunoprophylaxis, namely, the synthesis of drugs against pathogens which have a large number of antigenic determinants (pneumococcus) or a highly labile antigenic structure (influenza virus).

The clear description of events underlying the function of various cellular and molecular mechanisms of the immune system aptly described in this book will undoubtedly arouse the keen interest of physicians. The book should be of special interest to those engaged in the continual search for effective new means of immunoprevention and immunotherapy of infectious diseases.

*Professor B.F. Semyonov*

# Preface

This book is an overview of the latest achievements by a group of Soviet immunologists, immunogeneticists, and chemists whose combined work for decades has concerned the most urgent problems of modern immunology. Many of the new developments in immunologic science were first reported by the authors of this volume in pioneering works that have won recognition world wide. An enumeration of one's own scientific merits without substantiation from the current world literature is meaningless, however. Nor can the contribution of a research team to a given body of knowledge be properly assessed without a firm understanding of the general principles governing the fields. For this reason, the most interesting works of Soviet and foreign immunologists are included here to provide valuable illustrative material and reflect the rapid progress in immunology. Our own recent investigations make up the core of the book.

The chapter "Dictatorship of the Lymphocyte" deals with the ability of lymphocytes, the key cells of immunity, to interact with stem cells, the main cells of haemopoiesis. This remarkable property of lymphocytes was first discovered in our laboratory. Apart from their crucial role as effectors and regulators of immune reactions, T-lymphocytes can significantly influence haemopoiesis. The lymphocytes that exercise control over stem cells are called T-differentiating ones. The problem of T-cells is extremely important, particularly in the therapy of various circulatory diseases, including cancer, as well as in the correction of immunodeficiencies and other disturbances, using transfusions of haemopoietic tissue cells.

When studying the regulatory role of the bone marrow in the development of immune responses, we discovered a new level of cell interactions related to mature antibody producers rather than to immunogenic precursors. At this level, the stimulating effects of bone marrow cells are expressed by a soluble mediator—a stimulator of antibody producers (SAP) both *in vitro* and *in vivo*. It seems likely that pure preparations of SAP will soon be used widely in everyday clinical medicine as potent stimulators of antibody synthesis. SAP may also be useful in large-scale production of synthetic antibodies against staphylococcal, streptococcal, and other microbial infections. We have also succeeded in obtaining reliable evidence of the endorphine nature of SAP, which promises to revolutionize routine approaches to the study of immunomediators. This line of our research is described in the chapter "Molecular Messengers of Immunity".

The last chapter, "Vaccines of Tomorrow", deals with a phenotypic correction of genetic control over immunity, a complicated, but, nevertheless, very exciting field of research. Combining our efforts with those of a group of chemists headed by Professor V.A. Kabanov, Corresponding Member of the USSR Academy of Sciences, we have attempted to devise entirely new principles in immunology, namely, the reversal of individuals genetically characterized as low responders into high responders. Such a development would serve as the basis for the creation of new vaccines against such infectious diseases as influenza, venereal diseases, and malaria. The first step in this direction is the synthesis of artificial macromolecules based on synthetic polymers bearing antigenic determinants. In these molecules, the antigens yield immune responses that are further amplified

by artificial carriers, resulting in a high response to the antigenic determinants, independent of an individual's immune response genes.

These three major directions of our research serve one ultimate goal—to gain insight into the action of each individual link in the immune system and to control its function directly.

Occasionally, of course, our results were disappointing or we drew faulty conclusions. Often, facts that seemed of great consequence at first proved to be of little importance. Some hypotheses failed to be confirmed experimentally; others were changed beyond recognition or became untenable. But regardless of what happened, we never abandoned our motto of "forge ahead". A large body of evidence and our collective intuition have helped us to formulate some possible new directions for further research. Our studies are still in progress, and if the problems discussed in this book arouse the reader's interest and curiosity or, perhaps, the desire to participate in their solution, we will consider our task fulfilled.

*R.V. Petrov*

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# Dictatorship of the Lymphocyte

In this chapter, we describe recent advances in the study of lymphocytes, the main cells of the immune system, with the emphasis primarily on the ability of these cells to interact with red and white blood cell precursors and regulate their activity. Functioning as "dictators", lymphocytes control the proliferation of haemopoietic stem cells, the key cells of the circulatory system, suppress or stimulate their growth, and thus determine the direction of their differentiation.

Lymphocytes play a crucial role in the protection of human and animal organisms against various infections. When transplanted into an alien organism for therapeutic purposes, lymphocytes evoke tissue incompatibility responses and reject hostile organs and tissues, displaying thereby a unique capability to change the intensity of immune reactions. The discovery of the remarkable ability of lymphocytes to interact with blood cell precursors and control their activity launched a new era in the study of their functions, which may eventually lead to deciphering of intimate mechanisms underlying various cell interactions at the level of the key cells of the immune and circulatory systems. Such research is oriented toward putting haemopoiesis under directional control and, subsequently, curing various circulatory diseases, including cancer.

The ability of lymphocytes to interact with haemopoietic stem cells and to control their proliferation and maturation was first revealed by the Soviet immunologists Rem Petrov and Liya Seslavina in 1967. In the USSR this was registered as a scientific discovery.

## Stem Cells Are the Key Cells of the Circulatory System

The blood of all mammals, including humans, is a solution rich in proteins, in which the cells of three major types, i.e. erythrocytes, leucocytes, and thrombocytes, are suspended. Erythrocytes are red and numerous (about 5,000,000 per 1 microliter, μl). It is the erythrocytes that give the blood its red colour. The main function of red blood cells is to transfer oxygen. Leucocytes, or white blood cells, ingest and destroy foreign particles entering the blood, e.g., microbes. There are much fewer leucocytes (4,000-10,000 per

1 μl) than erythrocytes. Thrombocytes, or blood platelets, are the cells of a thrombus—a blood clot formed as a result of a cut or an abrasion. Thrombocytes can arrest haemorrhage by making the blood coagulate. The number of thrombocytes in 1 μl of blood is 200 000-300 000. The cells of all the three groups are produced in the bone marrow, a haemopoietic tissue carrying precursor cells. Like the seeds that give rise to the stem and branches of a tree, these precursor cells proliferate, maturate, and ultimately evolve into blood cells "branching" into erythrocytes, leucocytes, and thrombocytes. One blood cell can generate thousands of them.

What is the total number of precursors, or "seeds", in the blood? Does every type of blood cells have its own "seeds", as has been believed by many scientists? Or is there a single, common precursor, as was postulated by the Russian histologist A.A. Maksimov at the beginning of the twentieth century? There was no agreement on these questions until 1961, when J. Till and E. McCulloch (Canada) invented a procedure that made it possible to determine the number of the "seeds" and to predict their further fate or, scientifically speaking, to establish their paths of differentiation into erythroid (erythrocyte growth), myeloid (leucocyte growth), or megakaryocyte (thrombocyte growth) colonies. A standard procedure generally used to count the number of "seeds" and follow the fate of their "sprouts" involves intravenous injections of bone marrow cells into lethally irradiated recipients, e.g., mice. As a result, multiple colonies formed by haemopoietic cells begin to appear in the spleens of the recipients. The greater the number of transplanted bone marrow cells, the more the colony growth in the spleens of the irradiated animals, i.e., the greater the number of "seeds", the more "sprouts" there will be (Figs. 1 and 2). If we cut the spleens containing the colonies of haemopoietic cells into very thin sections, stain them with special dyes, and put them under the microscope, we will be able to determine the number and type of these colonies. Of the total colony growth, 60% are erythroid, 30% myeloid, and 5% megakaryocyte colonies; the remaining 5% are practically indistinguishable. This ratio of 60:30:5 remains constant, irrespective of the number of bone marrow cells transplanted to the irradiated animals. This implies that increases and decreases in the number of colonies, or changes in their ratio as the result of various influences, are strictly controlled by stem cells, particularly those "germinating" as erythroid, myeloid, or megakaryocyte colonies.

This fact is of great importance for the identification of factors that control stem cell activity as well as for the quantification of this control. But the most surprising is the observation that after injection of erythroid cells, i.e. "seeds" germinating as erythrocytes, into the veins of lethally irradiated recipients, all three colony types will be formed in the recipients' spleens at the same ratio of 60:30:5.

Hence, one precursor can generate every type of blood cell and is thus common to them all. So A.A. Maksimov was correct in predicting the existence of a single precursor cell capable of giving rise to several histologically different colonies. Such cells, called multipotent cells, can be further transformed into blood cells of many types (erythrocytes, leucocytes, thrombocytes), but not into cells of a single type (monopotency). As we will see later on, multipotent

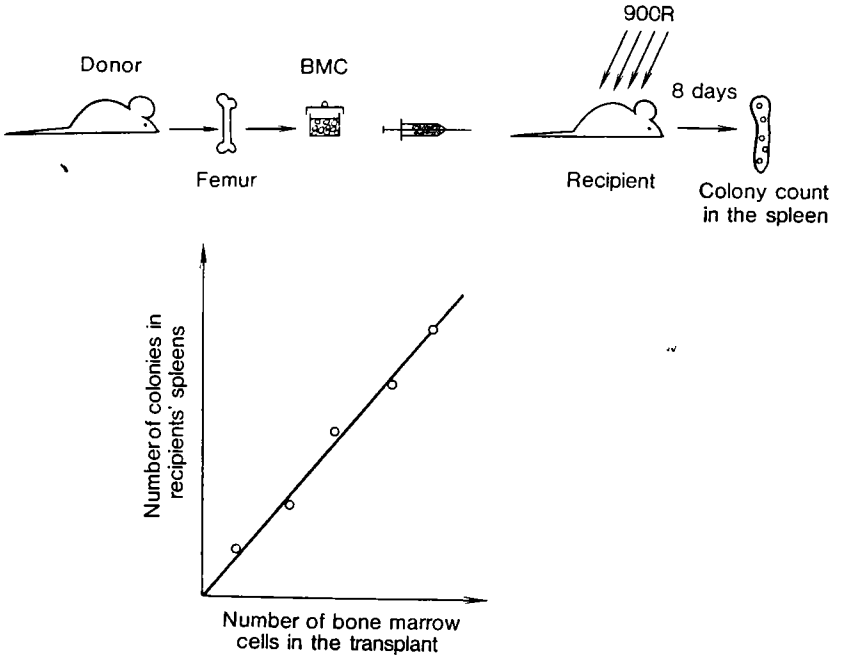



**Fig. 1.** Determination of the number of haemopoietic stem cells in murine bone marrow (diagram of principles).
BMC—bone marrow cells

precursor cells will further give rise to lymphocytes. Moreover, these cells are self-reproducible, and they can form new colonies within the organism of a lethally irradiated animal. In other words, when the precursor cells present among other cells of a colony are transplanted to the recipient simultaneously with them, they begin to produce new colonies, thus showing the tendency for self-reproduction. This is how the haemopoietic precursors got the name of stem cells: all "branches" of a haemopoietic "tree" shoot off the main "stem". Stem cells are the key cells of haemopoiesis; their main capabilities, i.e., self-reproducibility and multipotency, are realized in the course of their proliferation and maturation.

Thus, blood cells are derived exclusively from stem cells present in the bone marrow. Endowed with the ability to proliferate and maturate, stem cells maintain the constant number of erythrocytes, leucocytes, and thrombocytes in the blood. But why should stem cells localized in the bone marrow form colonies in the spleen and

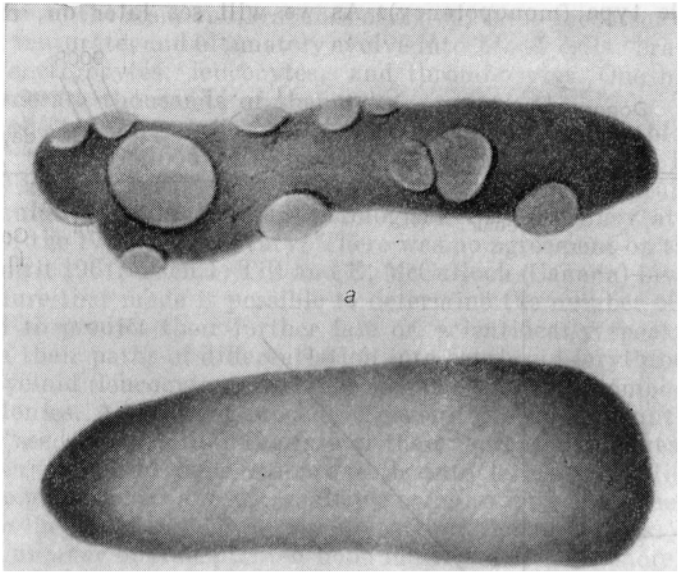



**Fig. 2.** Mouse spleens: lethally irradiated (*a*); intact (*b*) (several-fold magnification). Colonies of haemopoietic cells can be visualized (*a*)

not in the bone marrow cavity, the site of the bone marrow? And, if they do, are the colonies produced by stem cells in the bone marrow cavity distinct from those formed in the spleen? These questions are very important for a correct understanding of the events that will be discussed in subsequent chapter.

Stem cells, of course, can produce colonies in the bone marrow cavity. This capacity of stem cells can easily be illustrated by the experiment cited above (Fig. 1). Healthy bone marrow cells are transfused to lethally irradiated mice, but this time the colonies are counted in the femur rather than in the spleen. After special treatment, the femoral bone is dissected into thin longitudinal cuts that are further used for a microscopic colony count. In the bone marrow cavity, haemopoietic precursors are able to produce colonies of all

three types, i.e. erythroid, myeloid, and megakaryocyte, but at a ratio different from the splenic one. In the spleen, one myeloid colony is formed for every two erythroid ones, i.e., the correlation between the number of erythroid and myeloid colonies (the so-called E/M ratio) is 2:1. In the bone marrow cavity, the E/M ratio is 0.5:0.7, i.e., two myeloid colonies are formed for every erythroid colony. Therefore, in mouse spleens haemopoiesis is predominantly erythroid, while that in the bone marrow cavity is predominantly myeloid.

These data illustrate another important peculiarity of stem cells, namely, their ability to recirculate in the blood. When bone marrow cells are injected intravenously into lethally irradiated animals, stem cells begin to produce colonies in their spleens and bone marrow cavity. But this is not the unique property of the transplanted cells. In the spleens and bones of humans and other mammals, the haemopoietic tissue is arranged in foci. Stem cells that migrate from the bone marrow with the circulating blood communicate with other parts and organs of the haemopoietic system and are continually interchanged there. If necessary, these stem cells occupy the damaged areas in order to repair haemopoiesis there. The ability of stem cells to migrate from the bone marrow and recirculate with the blood is essential in the stabilization of the haemopoietic and immune systems. Later in this chapter, we shall discuss this in more detail.

This remarkable capacity of stem cells was revealed in a number of facts. Stem cells can always be detected in the blood of experimental animals. Blood cell transfusions from healthy to lethally irradiated animals save the latter from the inevitable death; as a result, thousands of erythrocytes, leucocytes, and thrombocytes produced by haemopoietic precursors, begin to appear in the blood of the radiation damaged animals. Moreover, normal blood cells injected intravenously into such recipients induce an intensive growth of erythroid, myeloid, and megakaryocyte colonies in their spleens. These colonies are practically indistinguishable from those produced by bone marrow cells injected intravenously into lethally irradiated recipients.

Before we come to the discussion of other data relevant to the ability of stem cells to migrate from the bone marrow and circulate in the blood, let us consider the following results.

Mouse cell nuclei contain 40 chromosomes of equal length. In late 1950's, a group of researchers from the Radiobiological Institute at Harwell (Great Britain) obtained a special strain of mice with translocation, i.e. partial transfer of the chromosomal material from one chromosome to the other, in the fourteenth chromosome pair. Under the microscope, the sixth chromosome pair of such mice appears to be unusually short (Fig. 3). This translocation was fixed, and the chromosome labelled the chromosome marker T6T6 (T stands for translocation, and 6 for the sixth chromosome pair). The mice bearing the natural marker differ from the parental strain only in the T6T6

chromosome pair. The use of the chromosome marker technique offers unique possibilities for cell typing, e.g., after immunization of irradiated mice with the blood or bone marrow cells of the T6T6-

**Fig. 3.** Mouse chromosome sets. Metaphase cell plates of CBA (*a*), CBAT6T6) (*b*), and (CBA × C57BL)$F_1$ (*c*)

labelled intact mice, the cells carrying shortened chromosomes begin to appear in their blood and tissues, thus leaving no doubt about their donor origin.

The Soviet immunologist Rakhim Khaitov modified and extended this procedure by combining the chromosome marker method with parabiosis, i.e., a cavitary conjoint of species. Earlier, it was shown

that two mice sharing common peritoneal tissues appear to have common circulation of the 2-4th day after surgery. This makes it possible to follow the translocation of labelled cells circulating with the blood from the body of one animal to the other. In this experiment, two identical mice were used, but one had a short T6T6 chromosome pair. The mouse devoid of the chromosome marker received a lethal dose of irradiation; the whole body of the second animal bearing the T6T6 label was also irradiated, but its hind limbs were cautiously screened with a lead plate. In this way, both animals appeared to be lethally irradiated, but the bone marrow of the second mouse remained intact. From now on, the further fate and behaviour of these surviving bone marrow cells—a potential source of stem cells—will become the object of our closest attention and investigation. Do you think that all this is reminiscent of a thrilling detective story, with an object under surveillance and an elegant, elaborate method of inquest that excludes, as a rule, every unforeseen move or alternative version? Either stem cells migrate from the bone marrow and circulate in the blood, or they do not. If they do, the spleens of mice bearing labelled chromosomes will contain colonies with the chromosome markers of mice whose bone marrow was protected by lead plate screening. If not, the colony growth will be absent in the spleens.

On the twelfth post-irradiation day, the colonies grown in the spleens of "non-labelled" mice were counted, and all of them contained the chromosome marker. Moreover, the T6T6-labelled proliferating cells were detected in other tissues, e.g., lymph nodes, bone marrow, thymus. This finding led R. Khaitov to the most plausible conclusion that the stem cells that migrate from the non-irradiated part of the bone marrow recirculate in the blood and eventually occupy the radiation-damaged haemopoietic and lymphoid tissues of the own and parabiont (i.e., surgically conjoined) organisms. There, the stem cells proliferate and produce multiple colonies, and thus provide for the regeneration of the damaged tissues.

Can one determine the rate of stem cell migration from the bone marrow? Attempting to answer this question, G. Hanks (USA) invented an original method that is schematically represented in Fig. 4. As in the above-cited experiments, the whole body of the animal was lethally irradiated, with the exception of the hind limb that was screened with a lead plate; therefore, the stem cells from this limb were the only ones to survive. While migrating from the protected part of the bone marrow, these cells circulated in the organism and occupied different tissues. Thus, the colonies that began to appear in the spleen on the 8-10th post-irradiation day could be produced only by the bone marrow cells from the intact limb. As you know already, stem cells migrate from the bone marrow, recirculate in the blood, form different colonies, and are self-repro-

ducible. But how can one determine their migration rate? Please have another look at Figure 4: a good knowledge of the prime properties of stem cells will make it easier for you to understand the further course of this experiment. One, two, four, six or seven hours after the first irradiation, it was repeated at the same dose (900 R), but this time the whole body of the animal was screened from head to tailtip (which is also a source of stem cells!), and only the hind limb previously protected by a lead plate was exposed to penetrating radiation. As a result of two irradiations, the whole body of the

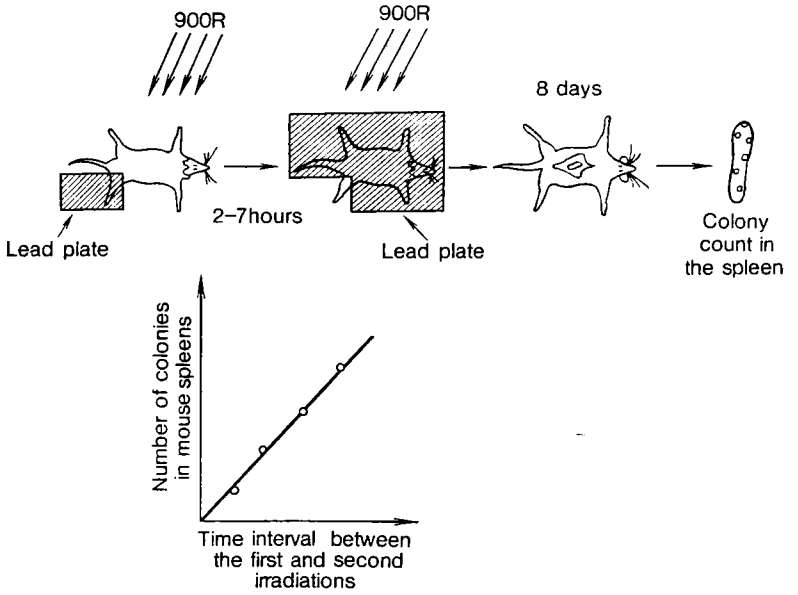



**Fig. 4.** Determination of the rate of stem cell migration from the bone marrow (diagram of principles)

mouse appeared to be lethally irradiated, but the stem cells that had migrated from the protected part of the bone marrow escaped death, and thus were "cut off" from those affected by the radiation. The "extruded" cells circulated within the organism; their content was determined in the spleen on the 8th post-irradiation day. The more the time interval between the first and second irradiations, the greater the number of stem cells migrating from the hind limb to the spleen to form colonies there. Knowing the number of colonies formed by stem cells in mouse spleens per unit of time, one can easily calculate the rate of their migration from the bone marrow, which is equal to 1.8 per hour. Assuming that in the above experiments the protected bone marrow cells made up to only 5% of the total mass

of haemopoietic tissue, the migration rate appears to be 36 stem cells per hour which, expressed on a daily basis, makes up to 860 precursor cells per 24 hours. The spleens of mice similar to those used by G. Hanks contain 11 colony-forming stem cells per every 100,000 bone marrow cells. Of the total number of bone marrow cells of approximately 400,000,000, about 44,000 are colony-forming stem cells. Taking the known value of stem cell migration equal to 860 per 24 hours, we obtain that every 24 hours about 2% of bone marrow stem cells are released into the blood flow.

Of course, the rate of stem cell migration varies widely in different individuals of the same species, to say nothing of different mammalian species, and it depends on the number of factors, such as circadian and seasonal changes, physiological state of experimental animals, their hormonal balance, etc. But even a perfunctory analysis gives an idea of high migration potentials of stem cells.

When studying the mobility of haemopoietic stem cells in different mammalian species, G. Strelin (USSR) found that the mobility of murine stem cells is much greater than that of rats and monkeys. In experiments conducted by this author, the bone marrow was also protected by lead plate screening, but the mortality in rats and monkeys irradiated under conditions similar to those described above was much higher than that in mice. Such species-specific differences in the survival rates after irradiation may be due to differences in the rates of stem cell migration from the protected part of the bone marrow. If that were true, the stem cell "extrusion" from the protected part of the bone marrow would significantly increase the survival value. To test this possibility, G. Strelin employed an original approach (autotransplantation) that can further be used in the clinic. Bone marrow cells from the screened part were intravenously injected into the same animals, i.e., were autotransplanted. The survival value increased markedly, thus suggesting that stem cells of rats and monkeys are far less mobile than those of mice. Extruding stem cells from the bone marrow, e.g., by autotransplantation, one can considerably increase their migration in the organism and provide for a better protection from penetrating radiation.

## Lymphocytes Are the Key Cells of the Immune System

Lymphocytes, or lymph cells, make up to one third of the total amount of white blood cells. But they originate from the lymphoid organs and not from the bone marrow. The lymph outflowing from all human tissues via lymphatic vessels enters the lymph nodes to get a new supply of lymphocytes. The lymphatic vessels that connect numerous lymph nodes come together into a large vessel, the thoracic duct, which drains into the blood near the

heart. In this way, lymphocytes pursue their course—from lymphoid organs to the blood, from the blood to tissues, and again into the blood.

T- and B-lymphocytes can travel in the body from several days to several months, depending on their lifetime. Unlike other blood cells, e.g. erythrocytes, that are passively conveyed with the blood, lymphocytes have their own peculiar way to return to the lymphoid organs: their route lies through the so-called post-capillary lymph node venules. On their way, lymphocytes not only push through the intercellular spaces, but permeate the cell cytoplasm of the venules without doing any harm to the vascular walls. In a lifetime, every lymphocyte travels at fantastically long distances—from 100 to 1,000,000 kilometres, and thus can be assigned to the most mobile cells of the body. In contrast with erythrocytes that never leave the blood stream, lymphocytes migrate from the blood to the tissues, then to the lymphatic ducts and again emerge in the circulating blood. They scour every "back street" of the body in search of mutant or cancer cells.

All genes are known to be subjected to occasional changes, or mutations. A cell containing a mutant gene, becomes a mutant. Mutation is a rare event; nevertheless, in every large cell population one can always find several mutants. Their incidence rates about $10^{-6}$. In a human organism, there are $10^{13}$ cells; therefore, at any moment the number of mutants can be as high as $10^{7}$. Ten million cells with changed, and, perhaps, noxious properties! Ten million cells ready to proliferate and do the work harmful for the organism, threatening with cancer or other malignancies. Surely there must be someone who will make short work of these "traitors", but who could that be? The answer is unambiguous: it is the immune system and its central figure, the lymphocyte. Blood lymphocytes and lymphoid organs constitute the crack troops in the army of immunity that stand on guard of the genetic equilibrium of the organism and repel invaders of any kind, be it microbes, viruses, or cancer cells. A foreign cells, or a transformed lymphocyte is immediately recognized by lymphocytes that instantaneously trigger off an immune response and demolish the "traitor", or the impudent invader. Lymphocytes are used to come as winners out of this battle, but sometimes their victory is frustrating for the organism.

Transplantation of bone marrow cells is by far the most effective procedure used in the therapy of circulatory disturbances of various etiology, e.g., congenital defects in the maturation of stem cells, cancer, radiation injuries, etc. As we have seen, the bone marrow is a haemopoietic tissue. During transplantation of haemopoietic cells, deficient or destroyed (e.g., by irradiation) stem cells of the recipient are substituted for by viable elements which supply the injured organism with healthy blood cells. Today, no other medicinal pro-

cedure stands comparison with bone marrow transplantations from the point of view of efficiency.

In reality, such ideal situations are observed only when bone marrow cells are transplanted between genetically identical species, e.g., enzygotic twins or specially selected animal strains, in which all individuals are exactly alike and are virtually genetic copies, i.e., between biologically indistinguishable twins. In other cases, the therapeutic effect fully depends on the mode of interaction between genetically different cells of the transplant and those of the recipient. The most active participants in this interaction are bone marrow lymphocytes. Interacting with one another and with the transplanted cells, lymphocytes function as "killers". They are programmed to recognize their "self" and to kill the "non-self" elements: this is their job, their predestination. Within the recipient's organism, transplanted lymphocytes open hostilities against "native" cells: they interact with stem cells and deprive them of the ability to proliferate and maturate. This is more than a mere hand-to-hand fight, this is a war to the death.

Indeed, the bone marrow transplanted with one purpose—to work a cure—appears to be a Trojan horse carrying hordes of killers with olive-branches in hand. But even this dramatic battlepiece does not fully reflect all the exciting peripeteia of the fierce assault the transplanted bone marrow cells have to withstand once they have trespassed on the territory of a "foreign state". The point is that the number of lymphocytes in the recipient's organism is much higher than that in the graft. We have already quoted the astronomical figures relevant to lymphocyte content in various lymphoid tissues. Apart from the bone marrow, lymph, and blood, lymphocytes can be also found in other organs, such as spleen, thymus, lymph nodes, Peyer's patches, tonsils, appendix, and adenoids. Lymphocytes protect the organism from all sorts of microorganisms, unrelated proteins as well as from their own transformed cells, or those transplanted as a suspension or a solid graft (e.g., skin, kidney, heart, bone marrow, etc.). Like bone marrow cells of the transplant, recipient's lymphocytes are committed to recognize their "self" and wipe out every "foreigner" encroaching on their territory. Transplanted bone marrow cells are no longer "self", even if obtained from the "next-of-kin", and they are immediately recognized by the recipient's lymphocytes, regardless of how great their similarity would be. Once the transplanted bone marrow cells (and, accordingly, stem cells) are rated as hostile elements, they are doomed to death. Sooner or later they will be destroyed by "native" lymphocytes, and the organism will be thus deprived of the life-giving force encased inside the healthy cells.

Furthermore, endeavouring at a total and unconditional victory in the battle-field, lymphocytes begin to reinforce their rears. As we

know, stem cells maturate into functionally different blood cells, in particular, into leucocytes whose main function is to ingest microbes, foreign or mutant erythrocytes, and other cells or cell fragments, and to destroy them using a large set of special enzymes. Phagocyting leucocytes give lymphocytes a helping hand in the struggle for the sovereignty of their "state" and help them to remove the killed ones. Besides "non-self" cells, lymphocytes can interact with their own "self", but they treat them in a different way. In wartime, i.e., after being transplanted into a foreign organism, lymphocytes switch the maturation of stem cells towards a hyperproduction of phagocyting leucocytes. Perhaps it is necessary, but the bone marrow cells are transplanted in the hope that within the recipient's organism, they normally maturate and eventually replace the damaged or destroyed cells. Alas, this does take place, but only for the time being. In the recipient's organism, some stem cells are killed by hostile lymphocytes, while others are impelled by their own lymphocytes to change the direction of their maturation and thus cannot fulfil their main task. Lymphocytes win in this combat, but their victory is rather a defeat for the organism and, in the first place, for the lymphocytes that counteract stem cells with a zeal that costs them life.

What is going to happen next? Is it the end of the story? Not at all, the main thing is yet to come. Every passing moment the surrounding world poses new questions and offers ways to their solution. Let us imagine, for a moment, that we are visiting a hospital. Some of its patients suffer from stem cell anemias caused by overproduction of erythrocytes, others have leukemias, i.e., impaired white blood cell formation. Quite a few are stricken with various immune diseases, etc. Now let us suppose that we have disclosed and made ourselves familiar with lymphocytes capable of interacting with stem cells and learnt how to use them for governing the maturation of stem cells and for changing the differentiation of haemopoietic precursors in any desired direction. Now, in case of emergency (e.g., microbial infections in the post-operative period), we may "invite" lymphocytes to switch stem cell differentiation toward a predominant formation of phagocyting leucocytes. In other situations (e.g., after transplantation of bone marrow cells to irradiated recipients or to recipients with various bone marrow deficiencies), we may even "order" lymphocytes to assist stem cells in repairing haemopoiesis—lymphocytes can do that too. But if the maturation of stem cells proceeds in a way that is dangerous for the organism, we would rather advise lymphocytes to neutralize these stem cells, so as to relieve the danger and make the organism healthy again.

Occasions are numerous when the interference of lymphocytes in the work performed by stem cells becomes expedient. But as a matter of fact, we still do not know how to control the stem cells function. In order to be able to do it, we must, first of all, delineate the relation-

ships between lymphocytes and stem cells, put the activity of lymphocytes under directional control and investigate, in every detail, their interactions with stem cells, so as to avoid pitfalls. Then, we must learn to discriminate between different types of lymphocytes, i.e., to find out "who is who". Only then can we draw a line between lymphocytes capable of interacting only with stem cells and those interacting with other lymphocytes. The next step is to find out whether or not the lymphocytes that interact with stem cells are capable of producing antibodies, destroy other cells, or control other lymphocyte activities, or whether or not the functions mediated by lymphocytes of different types are universal or unique. To answer the "who is who" question adequately is very important for the correct understanding of functioning of the whole immune system. For lymphocytes, the key cells of the immune system, play a crucial role, protecting the organism from foreign invaders as well as from occasional "treachery" of their own "brothers-in-arms" that give way to outward pressures.

In order to steer the work of individual components of a system and, accordingly, of the whole system, one has to get a thorough knowledge of it. Very often, the immune system is likened to a machine, being referred to as the "mechanism of immunity". Possible solutions to the problem lie in the identification of lymphocytes capable of interacting with haemopoietic stem cells among the already known ones or, if necessary, in the discovery of novel types of lymphocytes capable of such interactions. Studies in the field serve one basic goal—to bring lymphocytes and, eventually, haemopoiesis under directional control.

## Origin of Lymphocytes

Lymphocytes, the main cells of the immune system, are localized in the blood and lymphoid organs that together constitute the organ of immunity. This is a collective organ which, unlike many other organs situated in one locus, e.g., heart, lungs, is scattered all over the body (Fig. 5). The thymus lies at the base of the neck, right behind the sternum; Peyer's patches are attached to the inner surface of the small intestinal wall; the bone marrow fills the cavities of bones. Lymph nodes are diffused throughout the organism, and they are subdivided into popliteal, inguinal, submaxillary, subaxillary, mesenterial, etc. The spleen is also a lymphoid organ. Adenoids, tonsils, and appendix abound in lymphocytes. Although dispersed, the constituitive organs of the lymphoid system are associated through a vast network of lymphatic and blood vessels, and they function as a single whole by virtue of a continual exchange of lymphocytes. At an encounter with foreign proteins (antigens) or

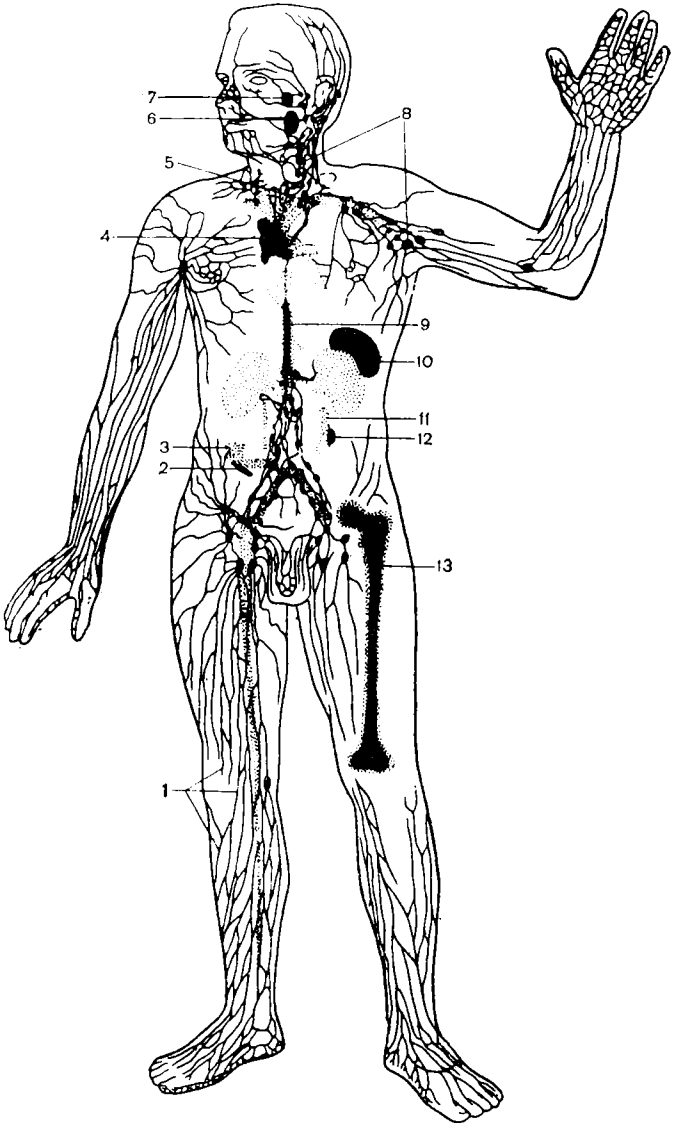



**Fig. 5.** The lymphoid system of man:

*1*—tissue lymphatic vessels; *2*—appendix; *3*—large intestine; *4*—thymus; *5*—left subclavian vein; *6*—tonsils; *7*—adenoids; *8*—lymph nodes; *9*—thoracic lymphatic duct; *10*—spleen; *11*—small intestine; *12*—Peyer's patches; *13*—bone marrow

their own mutant cells, lymphocytes, together with macrophages, or phagocytic cells, yield an immune response whose power characterizes the reactivity of the organism to a particular antigen. The immune response of an organism to a particular immunogen is always specific. Some individuals can develop high responses to one antigen (e.g., microbe), and low responses to another (e.g., serum albumin), while the responses of other individuals to the sum antigens may be quite opposite. In other words, there is no uniform immunologic reactivity. Immune responses of different individuals to the same antigen, or those of the same individual to different antigens vary within very broad limits. The whole complex of functions fulfilled by the cells of lymphoid organs is a functional criterion of the whole immune system. The manifestation of immune responses, i.e., their level, is controlled genetically. The genes that control the intensity of immune reactions are called Ir-genes (from "immune response").

According to their functions, lymphocytes engaged in immune reactions can be classified into two large groups, or populations, i.e., T- and B-lymphocytes. T-lymphocytes are derived from the thymus (T stands for "thymus"), and are sometimes called thymus-dependent lymphocytes. These cells mediate various cell-type reactions: they "attack" tumours, reject tissue and organ grafts, evoke delayed type allergic responses, and protect the organism from viral infections. Sensitized T-cells generated in response to antigenic stimulation conduct different cell-type effector functions. B-lymphocytes owe their name to the bursa of Fabricius, a lymphoid organ situated on the dorsal surface of the avian cloaca that serves as a diverticulum. A surgical removal of this organ leads to selective suppression of antibody synthesis as well as to cessation of humoral immune responses induced by B-lymphocytes. T-cell-mediated immune reactions are unaffected by this surgery. In man, no analog of the bursa of Fabricius has been found so far; there is some evidence that its closest counterpart is the bone marrow. T- and B-lymphocytes form independent systems that differ both in origin and in the type of immune responses (i.e., cell-type and humoral) mediated by these cells.

All lymphocytes as well as blood and phagocytic cells have a common precursor—a haemopoietic stem cell which gives rise to a lymphoid stem cell. Unlike the former, lymphoid stem cells do not evolve into erythrocytes, leucocytes, or thrombocytes; instead, they generate several intermediate types of lymphocyte precursors, i.e., T- and B-lymphocytes (Fig. 6). Haemopoietic and lymphoid stem cells, as well as B-lymphocyte precursors, inhabit the bone marrow; T-lymphocyte precursors, however, are expelled from the bone marrow and are released into the circulating blood. Migrating with the blood, T-cell precursors ultimately reach the thymus, the main organ of immunity. There, in the wholesome atmosphere created by

epithelial cells and humoral factors, T-lymphocyte precursors further develop into mature T-cells.

The rate of lymphocyte proliferation in the thymus is very high. Although the thymus makes up to only 10-15% of the total mass of lymphoid tissue, it generates up to 50-65% of all lymphocytes produced by the organism within 24 hours. Like other lymphoid organs, the thymus is a constituitive organ of the circulatory system. An

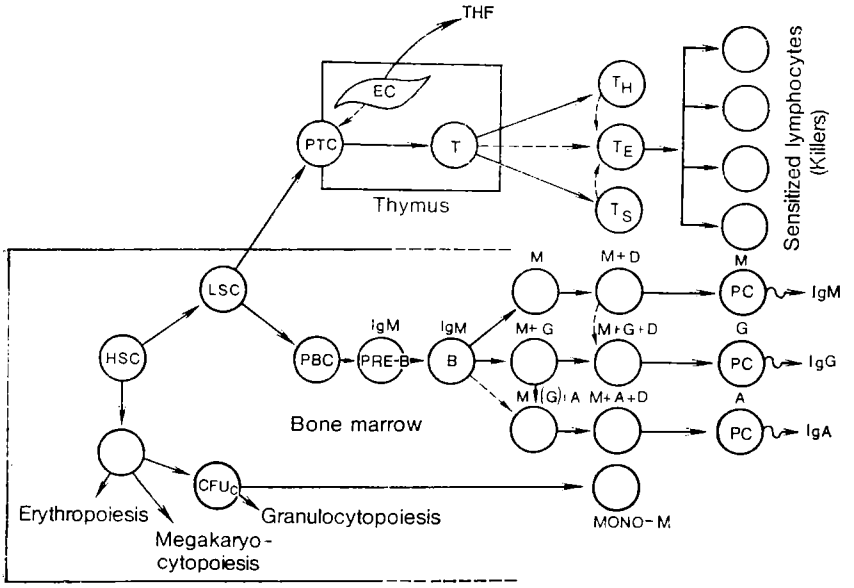



**Fig. 6.** Development of immunocompetent cells (WHO, 1977): HSC—haemopoietic stem cell; $CFU_C$—colony-forming unit in culture; LSC—lymphoid stem cell; THF—thymus humoral factor; T—T-lymphocyte; $T_H$—T-helper; $T_E$—T-effecto; $T_S$—T-suppressor; PBC—precursor of B-cells; PRE-B—pre-B; B—B-lymphocyte; PC—plasma cell; M, D, G, A—immunoglobulin molecules of various classes (IgM, IgD, IgG, IgA); MONO-M—monocyte-macrophage

interesting property of the thymus is that T-cells that are being continually ousted from this organ can never come back again, for the front gates of the thymus are now shut for them forever. Colonization of the thymus is a privilege of stem cells with a bone marrow "passport". A complete renewal of the thymus population due to the inflow of stem cells and the outflow of T-lymphocytes formed from them, normally takes 4 to 6 days. Another important peculiarity of the thymus is that only 5% of the newly formed T-cells are released from the thymus into the circulating blood; for the overwhelming majority of these cells the thymus becomes a cemetery: after 3 or

4 days these cells die there. The reason for their death is not completely understood.

A standard procedure commonly used for separation of mature T-lymphocytes from immature ones is the injection of mice with the hormone hydrocortisone. Two days after the hormone injection, more than 90% of T-lymphocytes leave the thymus, but the remaining 10% are worth a hundred such. These are cortisone-resistant, mature, long-living cells localized in the medullary layer of the thymus; they are more radiation-resistant than immature, cortisone-sensitive T-lymphocytes that are predominantly localized in the thymus cortical layer and that reveal opposite properties.

T-lymphocytes which are expelled from the thymus, are carried with the blood to lymph nodes, spleen, or tonsils; a small part of them occupies the bone marrow or the thoracic duct. Inside the lymphoid organs, T-lymphocytes settle down, strictly in order, in specially allotted localities, called T-dependent zones (Fig. 7). Here, the T-cells further ripen in peace, supporting themselves on the humoral factor secreted from the thymus into the blood. This is why T-lymphocyte precursors leave so hurriedly the bone marrow for the thymus: the organism has to be reinforced with a fresh supply of "warriors" capable of recognizing and destroying "foreign invaders" or their own transformed cells before they develop into malignant ones. T-lymphocytes that make up to 80% of the total army of lymphoid cells, safeguard the genetic equilibrium of the organism. A removal of the thymus causes various immunodeficiencies, and after a while (usually from several weeks to several months), a death from an infectious disease or a malignant tumour ensues.

Lymphocytes which take their origin from T-cells can be split functionally into three main groups, or subpopulations, i.e., T-helpers, T-suppressors, and T-effectors, which differ both in "appearance" and in the functions they mediate. The first two groups (i.e., T-helpers and T-suppressors) are assigned to the category of regulatory cells. T-helpers induce B-lymphocytes to begin antibody synthesis, while T-suppressors inhibit various immune responses and, if their activity is high enough, they may cause various immunodeficiencies, e.g., immunoglobulin deficiency—hypogammaglobulinemia, or the so-called immunologic tolerance (the lack of response to certain antigens). T-suppressors inhibit cell-type and humoral immune reactions. During malignant growth, the activity of T-suppressors increases manifold. Conversely, when the activity of these cells is low, the lymphoid system becomes hostile even to its own antigens, to which it is otherwise tolerant, eventually resulting in severe immunologic disorders generally termed as autoimmune diseases.

B-lymphocyte precursors generate several intermediate forms and ultimately evolve into bone marrow B-cells (Fig. 6). These cells migrate with the blood from the bone marrow to peripheral lymphoid

organs, where they occupy B-dependent zones (Fig. 7). Unlike T-lymphocytes which mediate cell-type immune reactions, B-lymphocytes engage in humoral immune responses via the synthesis of protective protein molecules—antibodies. In case of antigenic attack (e.g., microbial invasion), B-lymphocytes begin to proliferate and

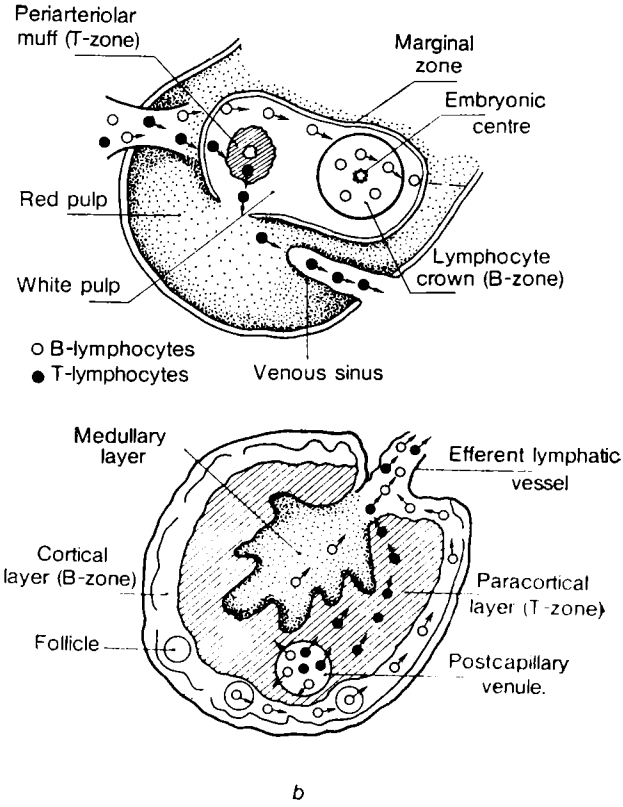



**Fig. 7.** Schematic presentation of spleen (*a*) and lymph node (*b*). The arrows designate the direction of migration of T- and B-lymphocytes.

differentiate into plasma cells which secrete specific antibodies, or immunoglobulins. These antibodies can be likened to magnetic mines that drift in the open sea and cling firmly to the bilges of passing-by ships with the view of destroying them and protecting the coastmen from the unbidden visitors. In much the same way, antibodies circulate in the blood about the organism, until they come across the microbes that evoked their formation and bring them to destruction with the help of a special set of blood serum

enzymes (the so-called complement) that adhere to the antibodies mounted on the surface of these microorganisms.

Antibodies can be directed not only against microorganisms, but against blood serum proteins, viruses, blood cells (e.g., erythrocytes, leucocytes), artificial antigenic molecules, etc. The wide use of antilymphocytic sera in laboratory practice has become a commonplace procedure.

Injecting pure T-cells (e.g., murine) into the aural vein of rabbits, one can obtain after 2 or 3 weeks large amounts of specific antilymphocytic antibodies against T-lymphocytes of the immunizing species, in our case, mice. In order to detect these antibodies, rabbit blood serum (complement) is added to mouse T- and B-lymphocytes. In the presence of the complement, T-lymphocytes are destroyed by the antibodies formed, while B-cells remain unaffected. This is a sign that the blood serum of immunized rabbits contains highly specific antibodies against T-lymphocyte antigens. In a similar way, one can obtain antilymphocytic sera that will specifically interact only with B- or even with all lymphocytes; in this instance, a mixture of T- and B-cells (e.g., from lymph nodes) is intravenously injected to rabbits.

Such antisera have proved to be convenient diagnostic tools that are widely used for identifying lymphocytes (T or B), or analyzing their roles in different immunologic processes. At present, antilymphocytic sera are being gradually displaced by homogeneous preparations of monoclonal antibodies produced by specially selected cell lines—hybridomas.

The immune reaction, besides effector cells, involves the so-called memory cells that store and spread within the immune system the information about antigens. Lymphocytes, the carriers of immunologic memory, live for about 20 years, sometimes even longer. In the absence of antigens that evoked their formation, lymphocytes are inert, and they travel leisurely about the body. But the moment the antigens reappear in the blood, the lymphocytes that keep them in memory, trigger off immediately a secondary immune response that is faster and stronger than the primary one. Similar to the central nervous system, the immune system enjoys an acquired memory that is not inherited.

Another cell derived from the haemopoietic stem cell precursor is the macrophage. Macrophages are present in many tissues, e.g., lymphoid organs, lungs, liver, nervous tissue, etc. Together with phagocytic cells of the blood (monocytes) and their precursors, macrophages form a system of mononuclear phagocytes previously termed as the reticulo-endothelial system. In 1972, a group of WHO experts suggested the term "mononuclear phagocyte system", which is still in current use. In contrast with lymphocytes, macrophages do not originate from the lymphoid stem cell; they derive from a pre-

cursor they share with the granulocyte branch of haemopoiesis. As we have seen in previous sections, blood leucocytes formed from granulocyte cell colonies can also function as phagocytes: they ingest foreign cells or fragments of their own cells, microorganisms, etc., and decompose them with the help of intrinsic enzymatic systems to separate molecules that are further excreted from the organism. Apart from their clean-up function (phagocytes are often called "scavengers"), phagocyting cells, or macrophages, deliver antigen molecules to T- and B-lymphocytes that get them involved in various immune reactions terminating in their destruction and subsequent excretion.

## Functions and Membrane Structures of Lymphocytes

Until middle 1960's, lymphocytes, the key cells of the immune system, were regarded as integral cells involved, besides various cell-type immune responses, in the synthesis of protective protein molecules, or antibodies. These conceptions were based primarily on the changes occurring in lymphoid cells *in vivo*, e.g., during antibody synthesis or upon rejection of tissue grafts (skin, kidney, etc.). As an example, we may cite the response of the whole organism to a microbial antigen, e.g., *Leptospira*.

*Leptospira* belongs to the zoonotic group of microbes that transmit animal diseases to man and are communicated, for the most part, in aqueous media. *Leptospira* is one of the causes of jaundice, a severe disorder concomitant with anemia, liver dysfunction and renal lesions. Let us consider a case when experimental animals are immunized with a non-pathogenic strain of this microbe, so as to avoid undesirable symptoms. Three or five days after the injection of *Leptospira*, numerous antibodies begin to appear in the blood of these animals. These antibodies adhere to the microorganisms circulating in the blood and agglutinate them; to detect them is not difficult. A drop of a microbial suspension of *Leptospira* is added to each of the test tubes containing different concentrations of antibodies (e.g., blood serum); a microscopic examination of these test tubes will offer a spectacular view. In a dark field (hence the name of this technique—"the dark field" method), numerous constellations of silvery "spiders" formed by the microbes agglutinated by the antibodies and thus made practically immobile, are visualized. Antibodies are produced in such immensely large amounts that even at high dilutions (e.g., when 1 ml of murine blood serum is dissolved in 5 l of an isotonic solution of NaCl) multiple silvery "spiders" will still be discernible against the dark background. At higher dilutions, the "spiders" disappear, and only occasional microbes can be detected microscopi-

cally. In this case, the antibodies, being too few in number, cannot agglutinate the microbes.

Such a hyperproduction of antibodies is the manifestation of a high immune response of the organism to the antigenic stimulation by microbes entering the body. The antibodies thus produced agglutinate microbes, make them immobile, and then destroy and expel them from the organism. By whom are these antibodies produced? Which of the cells can cope with such a huge amount of work? How are these cells incited to action? Who is the one to supervise this activity? What factors bring the antibody-producing plants to a standstill after the enemy has been disarmed and defeated? Specially stained histological slides provide no way to answer these questions in terms of routine histological approaches. Investigators from many laboratories sought to detect and describe subtlest structural changes in lymphoid tissues during the immune responses of the organism of various antigens (e.g., microbes, hostile erythrocytes, unrelated cells and proteins). These changes are always the same, i.e., predominant synthesis of mature secretory cells, the so-called plasma cells, having a well-developed cytoplasm and a potent protein-synthesizing apparatus. Plasma cells and their precursors, plasmo- and lymphoblasts, are characterized by enhanced synthesis of nucleic acids. The cytoplasm of these cells contains large amounts of specific antibodies. Within a few days following the antigenic attack, the number of antibody-producing cells increases 500-5,000-fold. Such a fantastic growth is the result of cell division; the sites of predominant accumulation of plasma cells have been established and characterized. However, in addition to transformed (plasma) cells, lymphoid tissues contain a great number of lymphocytes with unaltered structure. The percentage of cells involved in antibody synthesis is negligibly small—1%; the majority of cell changes are nonspecific. The intracellular shifts described above are typical of all lymphoid tissues and are not specific for one particular tissue.

Though essential, these data, however, do not provide satisfactory answers to the questions posed. Neither the antibody producers, nor regulatory cells turning on and off the "antibody production lines" can be detected at the level of the whole organism. Novel experimental approaches have to be devised for identification and clarification of the intimate molecular organisms underlying these processes. And yet, immunologists have found ways to overcome the difficulties and find clues to these questions.

Let us consider the following example. The lymphoid system of humans and animals is made up by two major types of lymphoid cells, i.e., T- and B-lymphocytes. T-lymphocytes mediate various cell-type immune reactions, while B-lymphocytes whose main function is to synthesize antibodies, engage in humoral responses. In our experiments, lethally irradiated mice in each treatment regimen

were divided into two groups, one immunized with T-cells mixed with an antigen (sheep erythrocytes), and one injected with B-cells mixed with the same antigen. To determine what kind of lymphocytes are produced by antibodies to the antigen the spleens were removed after a week, and the number of antibody-producing cells were determined. For this purpose, suspensions of spleen cells were placed on soft agar containing sheep erythrocytes, i.e., the antigen administered to lethally irradiated recipients together with T- and B-lymphocytes. As the agar hardened to form a jelly-like mass, sheep erythrocytes set inside it, but solute particles could freely penetrate through the intercellular spaces. After 1-1.5-hour incubation of the agar dishes at 37 °C, guinea pig blood serum (complement) was added to them. The mixture was let to stand for another hour and was then examined in transmitted light. Against the red background formed by the erythrocytes, small round erythrocyte-free zones could be seen, in which the erythrocytes had been destroyed by the antibodies formed by splenic antibody-producing cells in the course of incubation. Combining with the antigen that evoked their formation (sheep erythrocytes) and complement, the antibodies brought the erythrocytes to lysis, i.e., break-down. In this way, from the number of lytic zones, one may easily calculate the number of antibody-producing cells in the spleen.

While conducting this experiment, we were fully confident that the spleens of mice immunized with a B-lymphocyte—sheep erythrocyte mixture contained antibody-producing cells that were absent in the animals of the second group immunized with a T-cell—antigen mixture. With the same or similar stipulations, the immunologists from Melbourne (Australia) J. Miller, G. Mitchell and G. Nossal used for immunization of mice thoracic duct cells instead of T-lymphocytes, and bone marrow cells instead of B-lymphocytes. Great was the surprise of these investigators, when they came to a cell count. The mouse spleens in both experimental groups contained few or no cells capable of producing antibodies. Another experiment—the same results. In the spleen, the antibody-producing cells seemed to be nonexistent. So if antibodies are not produced by B-lymphocytes, who is their producer? Let us be patient and not be discouraged by these puzzling results. Our Australian colleagues, not at all disappointed, made a simple, elegant experiment that is depicted in Fig. 8. They included in the experiment an additional group of lethally irradiated mice and injected them with bone marrow cells mixed with thoracic duct lymphocytes and sheep erythrocytes. After a week-time, the results were assessed, and how amazing they were! In the third experimental group, the number of antibody-producing cells appeared to be 10 times as great as that in the first two groups taken together. In the first group animals injected with 1,000,000 thoracic duct lymphocytes, the average number of antibody-produc-

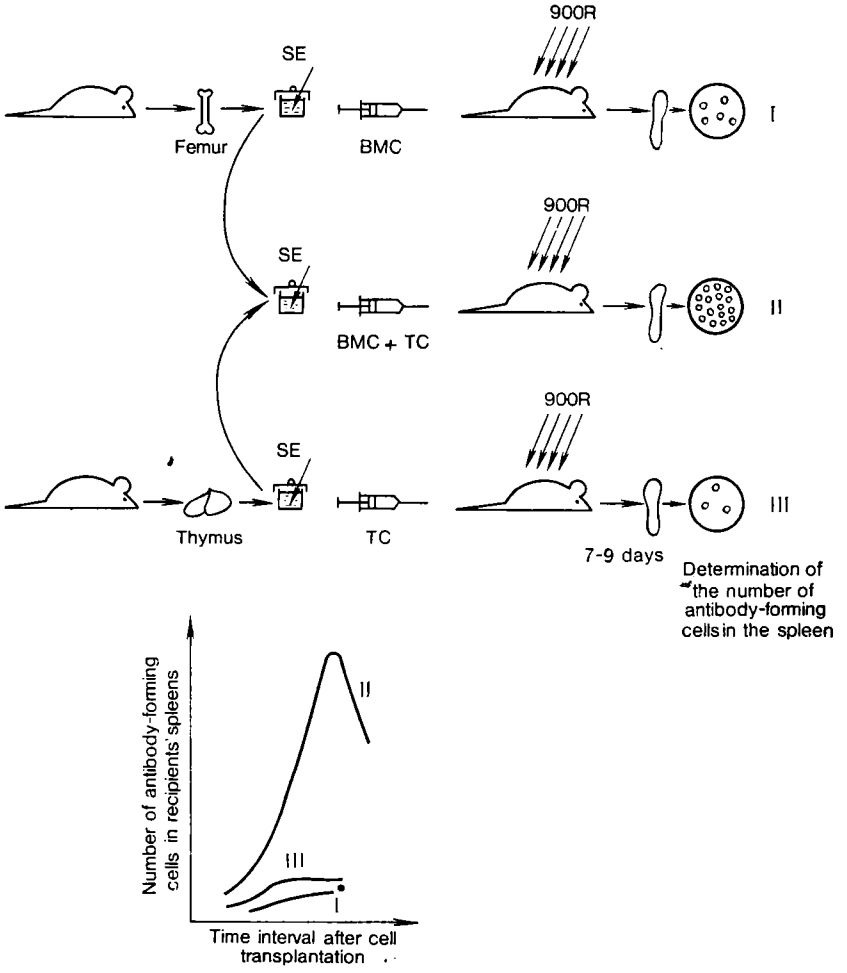



**Fig. 8.** Study of relationships between T- and B-lymphocytes during antibody synthesis.
SE—sheep erythrocytes; BMC—bone marrow cells; TC—thymus cells

ing cells in the spleen was 97, that in the second group animals injected with 10,000,000 bone marrow cells was 73. In the third group animals, to which 1,000,000 thoracic duct lymphocytes were administered in conjunction with 10,000,000 bone marrow cells, the number of cells secreting antibodies against sheep erythrocytes was 877! A more than 500% yield over the expected value (97 + 73 = 170)! Similar results were obtained, when thymocytes were used instead of thoracic duct cells as a source of T-lymphocytes.

Hence, the antibodies against the absolute majority of natural and synthetic antigens can be generated only by joint efforts of T- and B-lymphocytes; taken apart, they are fairly ineffective. Only upon simultaneous transfusions of these cells to lethally irradiated recipients could these lymphocytes produce protective immunoglobulin molecules. The feasibility of cooperative interactions between T- and B-lymphocytes was first postulated in 1966 by H. Claman *et al.* (USA). However, the assessment system employed by these authors was less explicit than that used by Melbourne researchers. Besides, H. Claman *et al.* failed to establish the true antibody producers. The clue to this problem came from experiments with T6T6-chromosome-labelled cells, carried out by J. Miller, G. Mitchell and G. Nossal, who succeeded in demonstrating that antibody-producing cells are generated by bone marrow rather than by thymus cells that seem to play a subordinate role. These results were first published in 1968, but they are still the most demonstrative evidence of the coordinative function of T- and B-lymphocytes in antibody synthesis and of the crucial role of B-lymphocytes in this process. T-lymphocytes have been assigned to a secondary part, i.e., to provide help for B-cells in antibody production.

Some antigens are thymus-independent, i.e., the antibodies against them can be raised in the absence of T-cells, e.g., in athymic animals or in pure B-cell cultures. To these one may relate microbial lipo- and polysaccharides (e.g., *E. coli*, a pneumococcal polysaccharide, $V_i$-antigen from *Salmonella*, etc.). The distinctive structural feature of these antigens is a rigid polysaccharide chain made up of tightly fitting, regularly alternating, repetitive antigenic determinants. The polysaccharide molecule thus appears as a multihapten "cartridge clip" formed by the macrophage for other antigens. An immune response to such multihapten molecules having a rigid linear structure is induced without the assistance of T-cells.

Analyses of the role of macrophages in the induction and realization of humoral immune responses led some authors to assume that antibodies are synthesized by more than two types of lymphoid cells. In 1969, simultaneously but independently of one another, R. Petrov (Moscow, USSR), I. Roitt and M. Berenbaum (London, Great Britain), and M. Richter (Montreal, Canada) postulated the existence of a three-cell immunopoietic system which includes T- and B-lymphocytes, and macrophages. Interactions between these three major types of cells stimulates the induction of antibody synthesis (Fig. 9). A more detailed description of molecular and intercellular events taking place at the initial steps of humoral immune reactions will be given in the chapter "Molecular Messengers of Immunity".

Thus, in late 60's, T- and B-lymphocytes, and macrophages brought up the rear of the main cell populations involved in the realization of humoral immune reactions. The same years were marked by the

appearance of pioneering works relevant to the multiformity of immunocompetent cells involved in the induction and development of cell-type and humoral immune responses.

As we know already, lymphocytes possess the ability to reject cell, tissue and organ grafts, and to destroy their own transformed cells, i.e. they can function as effectors.The discovery of T-cells playing an accessory role in antibody production demonstrated that lymphocyte populations (in this case, T-cell population) are functionally heterogeneous. One of the first to report on this phenomenon was A. Davies, a British immunologist, in whose laboratory the accessory function of T-lymphocytes in antibody synthesis was

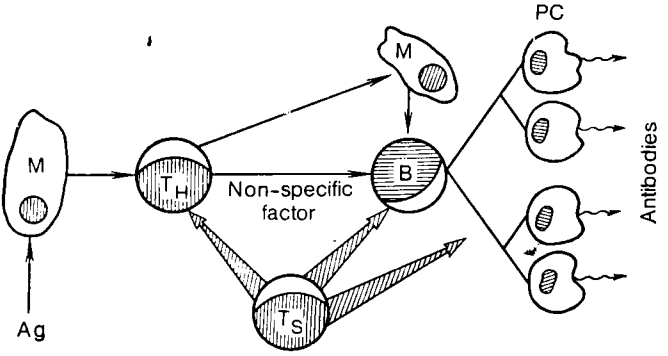



**Fig. 9.** Cell interactions during the induction of antibody synthesis. Ag—antigen; M—macrophage; $T_H$—T-helper; $T_S$—T-suppressor; B—B-lymphocyte; PC—plasma cells

established in 1967. The same results were reported a year later by the Australian researchers (J. Miller *et al.*); their experimental records were substantiated by simple and elegant reasoning as distinct from the cumbersome, far-fetched experimental scheme used by A. Davies *et al.* Obviously because of this their scheme did not become as popular as Miller's. However, the contribution of A. Davies and his group to the study of the accessory function of T-lymphocytes should not be overlooked or ignored. This author was the first to pave the way for further analyses of different lymphocyte subpopulations and to point out that besides killer lymphocytes, the T-cell population includes helper T-cells. Today we dispose of a more or less complete description of the T-system of immunity, which includes effector, helper, amplifier, and suppressor lymphocytes (Table 2).

The major function of T-effectors, i.e., the destruction of foreign or their own transformed cells was discussed in preceding sections. All other lymphocytes pertaining to the T-system are regulatory cells. T-helpers induce B-lymphocytes to begin antibody synthesis,

T-amplifiers stimulate the activity of T- and B-lymphocytes or stem cells, while T-suppressors inhibit other immunocompetent cell activities. In late 1970's, Soviet immunologists discovered two additional subpopulations of T-lymphocytes.

In 1978, Rem Petrov and Viktor Man'ko put forward a hypothesis according to which T-differentiating lymphocytes interact with haemopoietic stem cells by virtue of special receptors situated on their surface. Using these surface receptors, T-lymphocytes control the migration, proliferation and maturation of stem cells, and change the differentiation of haemopoietic precursors from the predominant erythroid to the predominant granulocyte growth. Do you remember the 60:30:5 ratio? Why, T-differentiating lymphocytes change it to

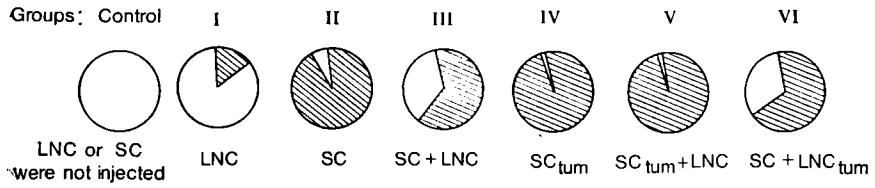



**Fig. 10.** Suppressor and countersuppressor activities of mouse lymphocytes. The hatched area corresponds to the degree of suppression of antibody synthesis in mice under effects of transplanted lymphocytes carrying suppressor or suppressor and countersuppressor cells.
LNC—lymph node cells of intact mice; $LNC_{tum}$—lymph node cells of tumour-bearing mice; SC—spleen cells of intact mice; $SC_{tum}$—spleen cells of tumour-bearing mice

0:90:5 by making haemopoietic precursors produce leucocytes instead of erythrocytes. Studies with these cells are currently under way, and some of them will be discussed in subsequent chapters.

Another T-lymphocyte subpopulation is represented by countersuppressor lymphocytes, whose main function is to block the suppressor activity of T-cells. Studies in the field were initiated in 1975 by S. Gambarov, R. Petrov, R. Khaitov, and A. Norimov who investigated the suppressor activity of lymphocytes in various murine tissues. Healthy animals were immunized with sheep erythrocytes, and the number of anti-erythrocytic antibody-producing cells in their spleens was determined five days thereafter. A week prior to antigen administration, the animals were injected with lymphoid cells with a presumed suppressor activity. It was expected that in tissues containing suppressor cells, the number of antibody producers would be low, while that in their absence would be unchanged. It appeared that the suppressor activity of lymph node lymphocytes in intact, nonimmunized animals was either very low or altogether absent. On the average, the number of antibody-producing cells in antigen-stimulated animals diminished by 16% as compared to the control group (group I, Fig. 10). After substitution of spleen cells for

lymph node lymphocytes (group II, Fig. 10), only 6% of cells retained their ability to synthesize antibodies; the remaining 94% were "neutralized" by T-suppressors.

These results were not unexpected. T-suppressors are known to be predominantly localized in the spleen; in lymph nodes, they are either scarce or absent. But the most striking was the observation that after simultaneous injection of the animals with spleen cells and lymph node lymphocytes (group III, Fig. 10), the number of active antibody producers did not decrease as might be expected, but rose drastically—from 6 to 40%. This is precisely the point: spleen cells contain suppressor lymphocytes, while lymph node lymphocytes are rich in cells that block the activity of these T-suppressors. That was an observation no one had ever made before. In another version of this experiment, spleen cells of intact animals were replaced by splenocytes of tumour-bearing mice (group IV, Fig. 10). The T-suppressor cells of these animals appeared to be far more active than those of intact mice; the inhibition of antibody synthesis was almost complete, i.e., 98.7%. However, the lymph node lymphocytes of intact mice did not eliminate the suppressor activity of lymphocytes obtained from neoplastic animals (group V, Fig. 10). In the mice immunized with a mixture of spleen cells of neoplastic donors and lymph node lymphocytes of intact animals, the percentage of antibody-producing cells was negligibly small—1%, the inhibition being 99%. Conversely, immunization of animals with spleen cells of intact mice and lymph node lymphocytes of neoplastic donors led to an increase in the number of antibody-producing cells from 6 to 40% (group VI, Fig. 10). These experiments have major theoretical and practical implications that may be summarized as follows.

1. Spleen cells of intact (healthy) mice contain suppressor lymphocytes that are either scarce or absent in lymph nodes (groups I and II, Fig. 10).

2. During malignant growth, the activity of suppressor cells enhances, while their ability to interact with other cells or receive their signals is markedly impaired (groups IV and V, Fig. 10).

3. Lymph nodes contain cells that eliminate the suppressor activity of lymphocytes. During malignant growth, their ability to interact with other cells or receive their signals is not impaired (groups III and IV, Fig. 10).

In 1975, the results of these experiments were published in *Doklady Akademii Nauk SSSR* (Proceedings of the USSR Academy of Sciences), vol. 224, No. 5, p. 1195, one of the leading scientific journals of our country. In 1979, Spartak Gambarov (Erevan, USSR) introduced a new term "countersuppressors", to describe lymph node cells capable of eliminating the suppressor activity of lymphocytes (S. Gambarov: "Countersuppressor activity of lymph node cells", *Armenian Biological Journal*, No. 8, p. 771, 1979). Two years later, countersuppressor cells

were described by R. Gershon *et al.* (USA) in a paper entitled "Countersuppression. A novel immunoregulatory activity" (*Journal of Experimental Medicine*, vol. 153, No. 6, p. 1533, 1981).

T-differentiating lymphocytes and countersuppressors conduct different functions: the former control stem cells, while the latter regulate the activity of T-suppressors. One common feature shared by these lymphocytes is that they both are regulatory cells.

Among the great diversity of T-cells, two more subpopulations can be distinguished, i.e., $T_1$- and $T_2$-lymphocytes, and they differ not so much by their functional capabilities, like other T-cell subpopulations (e.g., helpers, suppressors, killers, etc.), but are discriminated in terms of their lifetime, predominant localization, and ability to circulate in the blood.

The role of the thymus in cell-mediated immunity has been repeatedly emphasized in preceding sections. A surgical removal of this organ results in the elimination of cell-type immune responses which leads to severe disturbances in the whole immune system. In turn, T-immunodeficiency has a profound effect on the B-system of immunity, being concomitant with an elimination of T-cells which assist B-lymphocytes in antibody synthesis. Peripheral T-lymphocytes that gradually die off after thymectomy, can be classified into two groups, i.e., short- and long-living ones (Table 1).

**Table 1.** Properties of $T_1$- and $T_2$-Subpopulations of Mouse Lymphocytes

| Properties | $T_1$ | $T_2$ |
|---|---|---|
| Predominant localization | Spleen | Lymph nodes, thoracic duct, peripheral blood |
| Recirculation in the organism | Low | High |
| Predominant migration | To spleen | To lymph nodes |
| Lifetime | Short-living | Long-living |
| Sensitivity to low doses of antilymphocytic serum | Low | High |
| Irradiation resistance | High | Low |
| Elimination in adult animals after thymectomy | After 2-6 weeks | After 30 weeks |
| Elimination by thoracic duct drainage | Negative | Positive |
| Electrophoretic mobility | Low | High |
| Spontaneous proliferation | High | Low |
| Antigen Thy-1 content on cell surface | High | Low |

*Note*: Thy-1 (from "thymus antigen") is a specific marker for T-lymphocytes.

The distinctive features of $T_1$- and $T_2$-cells listed in Table 1 are often exploited in clinical practice for characterization of lymphoid cells involved in various immune reactions. Different experimental approaches are used for the purpose, and these include thymectomy (i.e., surgical removal of thymus) in adult animals, injections of antilymphocytic sera, irradiation of animals or cells with low radiation doses, treatment of lymphocytes with antisera against Thy-1, etc. Another routine approach is the immunization of lethally irradiated recipients with thymocytes and a subsequent removal of their spleens and lymph nodes for further analyses. The spleens of such animals predominantly contain $T_1$-, while their lymph nodes, $T_2$-cells.

Such an immense variety of functions is not typical of the T-cell population alone. Among B-lymphocytes, one can also find cells that can be classified into effector, helper, and suppressor subpopulations (Table 2).

B-lymphocytes that differentiate in response to antigenic stimulation (e.g., by microbes), evolve into antibody-secreting plasma cells that engage in humoral immune reactions. In this case, plasma cells function as effectors of the B-system of immunity. The antibody-producing cells thus formed are accumulated in peripheral lymphoid organs (spleen, lymph nodes, etc.).

B-helpers, another subpopulation of B-lymphocytes, carry out accessory functions. In 1977, studying T-effector interactions with genetically different stem cells and the role of B-lymphocytes in this process, V. Man'ko and T. Rudneva (USSR) demonstrated that B-lymphocytes do not destroy stem cells, but only assist T-cells in doing so; in other words, they function as T-helper cells. This assistance is reciprocal. T-cells help B-lymphocytes to synthesize antibodies and thus consummate their effector function; as for B-lymphocytes, they never leave this help unanswered and participate together with T-effectors in the induction of cell-type immune reactions. The occurrence of B-helpers in the B-lymphocyte population was confirmed by studies from many laboratories.

The assistant function of B-helpers in stem cell destruction by T-lymphocytes is not their single capacity. They help T-lymphocytes to generate suppressor cells. As we know, T-suppressors are regulatory cells that inhibit cell-type immune responses. In this way, B-lymphocytes not only help T-cells to destroy foreign cells, but also relieve them of doing unnecessary work. Apart from generating T-suppressors, B-lymphocytes can themselves develop a suppressor activity, especially on the territory of the bone marrow. Numerous works by R. Petrov, R. Khaitov, I. Sidorovich, and R. Ataullakhanov (USSR) demonstrated the crucial role of bone marrow B-suppressors in the elimination of humoral immune responses in this organ. B-suppressors inhibit the proliferation of antibody producer precursors and

**Table 2.** The Main Properties of T- and B-Lymphocytes

| Subpopulation | Properties |
|---|---|
| **T-lymphocytes** | Effector and regulatory cells of the T-system of immunity |
| T-effectors ($T_E$), antigen-specific and antigen-non-specific | Induce reactions of cell-mediated immunity (graft rejections *in vivo*, antitumour immunity, target cell lysis *in vitro*, delayed type hypersensitivity, etc.) |
| T-helpers ($T_H$), antigen-specific and antigen-non-specific | Involve B-lymphocytes in antibody synthesis |
| T-amplifiers ($T_A$) | Stimulate other cell activities (e. g., T- and B-lymphocytes, haemopoietic stem cells) |
| T-suppressors ($T_S$), antigen-specific and antigen-non-specific | Inhibit reactions of cell-mediated immunity |
| Countersuppressors ($T_{CS}$) | Eliminate the activity of T-suppressors |
| T-differentiating cells ($T_D$) | Interact with haemopoietic stem cells, control their migration, proliferation and differentiation |
| **B-lymphocytes** | Effector and regulatory cells of the B-system of immunity |
| B-effectors ($B_E$), $B_1$- and $B_2$-subpopulations | Antibody-producing plasma cells |
| B-helpers ($B_H$) | Stimulate the ability of T-cells to inactivate non-syngeneic haemopoietic stem cells, participate in the formation of individual subpopulations of T-lymphocytes ($T_S$) |
| B-suppressors ($B_S$), antigen-specific and antigen-non-specific | Inhibit proliferation of antibody producer precursors and other cells |
| **Lymphocytes devoid of T- or B-markers (third population lymphocytes)** | Mediate cell-type immune reactions |
| K-lymphocytes | Cause antibody-dependent lysis of target cells, including tumour cells, in the absence of complement |
| L-lymphocytes | Cause antibody-dependent lysis of some target cells in the absence of complement |
| NK-lymphocytes | Cause antibody- and complement-independent lysis of all nucleus-containing cells, especially of tumour cells |

prevent them from maturating into antibody-secreting cells. As a result, the bone marrow is relieved of immune reactions. In previous sections, we repeatedly emphasized the important role of the bone marrow as a single source of stem cells, the building blocks of the haemopoietic, immune, and mononuclear phagocyte systems. That is why this unique precursor-producing "plant" has to be safely protected from occasional breakdowns. The cells that watch and ward the bone marrow are B-suppressors.

B-suppressors are sometimes referred to as nonspecific cells. Their suppressor activity is not expressed toward a particular antigen, or toward the immune response challenged by it. Whoever the invader (a microbe or a foreign erythrocyte), these cells consider it their primary duty to nip in the bud any immune response evoked on their territory. Nonspecific B-suppressors are not ousted from the bone marrow to the spleen or lymph nodes; they just dare not desert their posts, and keep their domains under lock and key.

The bone marrow is not the single locus of B-suppressors. These cells can also be found in the spleen, lymph nodes, and peripheral blood. However, in these organs, B-suppressors are generated only in response to antigenic stimulation; their specificity is thus directed against the antigenic material that evoked their formation. For instance, in mice challenged with sheep erythrocytes, splenic B-suppressors inhibit the immune response to this particular antigen, but not to microbes or other immunogenic molecules. And vice versa, in the same recipients immunized with a microbial antigen, B-suppressors do not respond to sheep erythrocytes but are strongly stimulated by microbes. Any foreign substance entering the organism generates a B-suppressor which is specific for this particular antigen. This does not happen immediately after the antigenic attack, but after a certain time course, when the immune reaction is in full swing. Therefore, B-suppressors do not eliminate the induction or development of the immune reaction, but place certain constraints on them.

The multiformity of specific and non-specific regulatory cells is not a distinctive feature of the B-system of immunity. Antigen-specific and antigen-non-specific cells can be also identified within the T-lymphocyte population (effectors, helpers, suppressors, etc.). Whatever the individual properties of these subpopulations might be, they are functionally related. Antigen-non-specific lymphocytes are stimulated by any type of antigenic material entering the body; they are "omnivorous", so to speak. On the contrary, antigen-specific lymphocytes are challenged by one particular antigen.

The lymphoid system of mammals is not delimited by T- and B-lymphocyte populations. Recent studies have revealed quite a few lymphocytes which cannot be assigned to either of these categories (Table 2). These "odd" lymphocytes that can be subdivided

into two groups, K- and NK-cells, have the ability to kill target cells, for the most part, tumour cells. L-lymphocytes also pertain to this population.

K-cells (from "killer" cells) destroy target cells only when the latter carry on their surface a small quantity of antibodies specifically directed against their surface antigens. By virtue of these surface receptors which are sensitive to certain antibodies (their so-called Fc-fragments), K-cells bind to the antibodies fixed on target cells and bring them to lysis. The events preceding target cell destruction by K-lymphocytes are as follows. Within the organism, foreign or mutant cells function as antigens. Macrophage-treated products of target cell lysis (e.g., by macrophages, T- or NK-lymphocytes) are recognized by T-helpers which, by means of two signals, stimulate B-cells to begin antibody synthesis. In response to this call, B-lymphocytes begin to proliferate and ultimately evolve into plasma cells. The newly synthesized antibodies occupy the surface of undamaged target cells which evoked their formation and form a complex with them. K-cells bind by means of special receptors (Fc-receptors) to the antibody Fc-fragment within the antibody-target cell complex and bring the latter to lysis in the absence of the complement. K-lymphocytes constitute an alternative mechanism, which protects the organism from "nonself" elements that escaped death from macrophages or T- and NK-lymphocytes. This is, perhaps, the last defensive line of the body, for the participation of K-lymphocytes in cell-mediated immune reactions requires preliminary activation of both T- and B-cells.

NK-lymphocytes (from "natural killer" cells), in contrast with K-cells, destroy the "nonself" material at the very first encounter, unassisted by the antibodies or complement. NK-cells build up the first defensive line for foreign cells or transformed lymphocytes. Any nucleus-containing particle entering the body falls a victim to NK-cells; the latter are especially hostile to tumour cells.

K- and NK-cells originate from the haemopoietic stem cell; their immediate precursors, however, are still unknown. In some, but not all, features these cells resemble T- and B-lymphocytes. Presumably, these cells form an independent subpopulation of lymphocytes and are thus commonly termed as third population lymphocytes.

As for L-cells, they are very much similar to K-lymphocytes. The main function of these cells is to lyze target cells coated scantily with specific antibodies by binding via the Fc-receptor to the target cell-antibody complex. The distinctive feature of this subpopulation is that the L-cell Fc-receptor for immunoglobulin antibodies is rather thermolabile. With a rise in temperature from 4 to 37 °C, the Fc-receptor separates itself from the L-cell and passes into the environment. The lability of the Fc-receptor gives L-lymphocytes their name (L stands for "labile").

The enormous variety of lymphoid cells and cell populations provides for the highly effective performance of the multicomponent protective mechanisms of the organism as well as for the maintenance of their inner balance. Any transformed lymphocyte or a foreign cell or particle entering the body (e.g., with a bone marrow transplant) is immediately attacked by NK-cells. Simultaneously, B-lymphocytes switch on the "antibody-production lines" and generate sensitized T-lymphocytes. A certain tension mounts as regulatory cells—suppressor and amplifier T- and B-lymphocytes—join this process. The appearance of antibodies, even if in small amounts, is a guide to action for cytotoxic K- and L-lymphocytes to appear on the stage. As a result of such a fierce and unanimous counter-offensive, the harmful antigenic elements are destroyed and expelled from the organism.

Thus, the multiformity and polyfunctionality of lymphoid cells are provided for by the well-organized, highly mobile, coordinated protective systems of the organism. All their parts are effectively fashioned together, and every subdivision conducts a differentiated function. Some cells manufacture antibodies, others facilitate this process; the activity of both cells can be suppressed by other lymphocytes. Certain cells eliminate this suppression, kill foreign or mutant cells, or act as back-ups, and so on, and so forth. How can one discriminate single antigenic activities (e.g., helper or suppressor) among such a complex array, actually a cell babel, or at least tell T-lymphocytes from their B-counterparts?

One of routine methodological approaches used for identifying lymphocytes and their subpopulations is based on the analysis of macromolecular surface structures, with the help of which the cell recognizes the antigenic material (receptors), or which themselves are recognized by other cells (antigens). Certain surface antigens have the properties of cell receptors. The number of macromolecules lurking on the surface of lymphocytes is strikingly high. Some antigens and receptors of human T- and B-lymphocytes are shown in Fig. 11 with reference to mouse T- and B-cells. According to their distribution on the lymphocyte surface, these macromolecular structures can be classified into four main groups:

1. T-cell surface macromolecules: MTLA (mouse-specific T-lymphocyte antigen) and Thy-1 (thymus antigen) in mice and HTLA (human-specific T-lymphocyte antigen) and sheep erythrocyte-specific receptor in man. These structures are called T-lymphocyte markers, for they are specific only for T-cells.

2. B-cell surface macromolecules: MBLA (mouse-specific B-lymphocyte antigen) in mice and HBLA (human-specific B-lymphocyte antigen) in man. Immunoglobulin molecules can be detected both in human and murine B-lymphocytes. These structures are called B-lymphocyte markers.

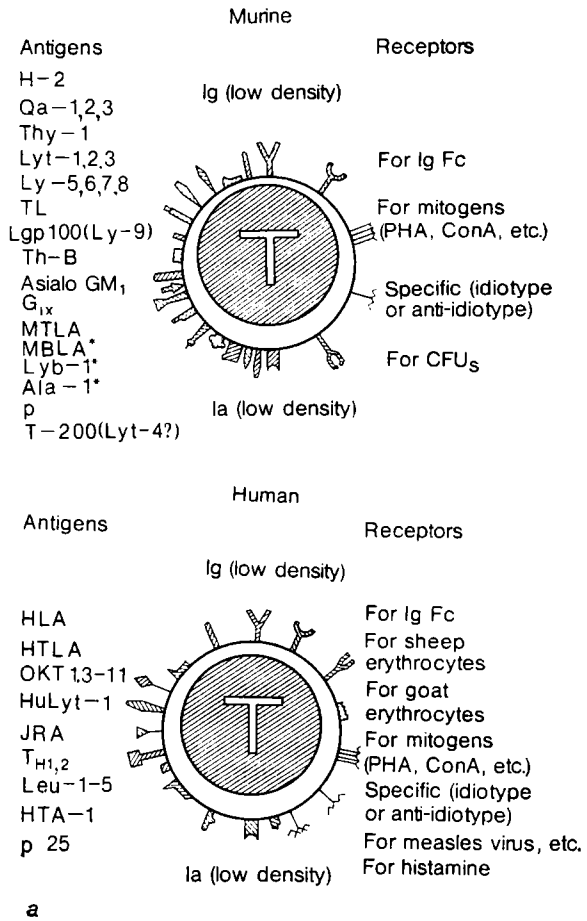
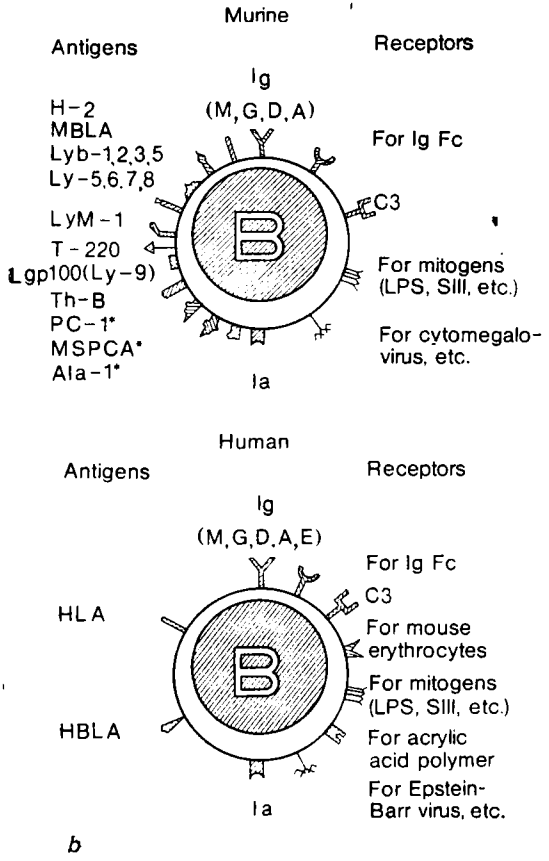



**Fig. 11.** Antigens and receptors of murine and human T-lymphocytes (*a*) and B-lymphocytes (*b*).
*On the activated ones

3. T- and B-cell surface macromolecules: histocompatibility antigens H-2 in mice and HLA in man. These antigens evoke tissue incompatibility responses upon transplantation. Other macromolecules assigned to this group are immunoglobulin Fc-fragment receptors, Ia antigens (I-region associated antigen) that participate together with immunoglobulin molecules in recognition of antigens and thus function as receptors, and Th-B antigen (thymus B-lymphocyte antigen) in mice.

4. Macromolecules specific for individual lymphocyte subpopulations.




*b*

The most conspicuous success has been achieved in the identification of surface macromolecules of various subpopulations of T-lymphocytes (helpers, suppressors, killers, etc.). Using this technique, T-cell subpopulations were identified with a great degree of accuracy not only in laboratory animals (e.g., mice), but in man as well. The "appearance" of T-killers and T-suppressors is very much similar; these cells differ primarily by their functional capabilities. Surface macromolecules of these two subpopulations are practically indistinguishable, but they differ markedly from those of T-helpers. The latter carry on their surface a Lyt-1 antigen, while T-suppressors

and T-killers carry a Lyt-2, 3 antigen. The same is true of human T-lymphocytes: T-helpers carry $T_{H1}$, T4, or Leu-3 antigens, while T-suppressors and T-killers are coated with $T_{H2}$, T5, 8 or Leu-2 antigens.

The macromolecules that are present on the surface of one cell subpopulation but absent on the other are called subpopulation markers. Mouse antigens Lyt-1, 2, 3, and human antigens $T_{H1,2}$, T4, 5, 8, and Leu-2, 3 are specific markers for T-helpers, T-suppressors, and T-killers, respectively.

The "portraits" of other lymphocyte subpopulations (i.e., counter-suppressors, T-differentiating cells, T-amplifiers, B-cells, third population lymphocytes) are less perfect. Studies with these lymphocytes were initiated not long ago, and they appeared to be very labour-consuming. It is not enough that a new macromolecule is detected on the lymphocyte surface which in itself is not an easy matter; it is necessary to prove its uniqueness and distinctness. Besides, one has to identify the lymphocyte subpopulation carrying this particular macromolecule (e.g., T, or B-, or third population lymphocytes) and only then come to analyze it at the subpopulation level. Surely, one can circumvent the difficult start by setting upon the distance midway or even near the finish, but sooner or later the path has to be traversed all the same. That is why the sketchy "snapshots" of individual lymphocyte subpopulations are not so impressive as the "group portraits" of the whole T- and B-families (Fig. 10). This problem demands further analysis and experiment, but even the first steps were marked by interesting and encouraging results. By way of illustration, we may cite a passage from the book by E. Gruntenko "Immunity: Pros and Cons" (Moscow, 1982).

Two Soviet immunologists, Yuri Malashkhiya and Gia Nadareishvili from the Institute for Advanced Training of Physicians at Tbilisi have devised a method which permits to detect T- and B-lymphocytes in small volumes of human cerebrospinal or intraocular fluids. This method provides a salient advantage over the existing methods of diagnosing some diseases of the central nervous system, in particular, of such severe disorders as tuberculous and serous meningitis. It was found that cerebrospinal fluid lymphocytes of patients with tuberculous meningitis are readily transformed into blastic forms and they vigorously proliferate in the presence of tuberculin. Lymphocytes of patients with serous meningitis function exactly in the same way as cerebrospinal fluid lymphocytes of healthy individuals. Therefore, by adding a few drops of tuberculin to a test tube containing cerebrospinal fluid lymphocytes of meningitis patients, one can diagnose the type of the disease and choose an adequate therapy. Until the introduction of this simple, sensitive method into clinical practice, no early differential diagnosis of serous or obliterative forms of tuberculous meningitis was possible.

It is becoming increasingly evident that clues to numerous problems of clinical medicine and applied immunology may come only from discrimination between different lymphocyte subpopulations, evaluation of their functional roles, elucidation of molecular and immunogenetic principles of their functioning, as well as from clarification of intimate molecular mechanisms underlying various cell interactions and a search for ways of directional control over lymphoid cells. All these objectives serve one ultimate goal—to repair haemopoiesis and regenerate the immune system.

## Lymphocytes Against Stem Cells

In the sections above, we defined the main properties of lymphocytes and stem cells, delineated their relationships and predicted the outcome of their interactions. Now it seems about time to make a more intimate acquaintance with lymphocytes that are capable of interacting with haemopoietic, in the first place, with foreign stem cells.

Until the appearance of pioneering works by R. Petrov and L. Seslavina in 1966, no one had seriously thought of bringing into one plane the lymphocyte, the main cell of the immune system, and the stem cells, the key cell of haemopoiesis. The real boom about cell interactions began two years later, with the discovery of the ability of T-cells to "order" B-lymphocytes to begin antibody "output". Yet, despite the explosive growth in the number of laboratories concerned with the study of T-B contacts, the most favoured field of research at the time, there was no question as to the feasibility of interactions between lymphocytes and haemopoietic stem cells.

Our initial studies in the field were conducted as follows. Two groups of lethally irradiated mice were injected with $10^6$ spleen cells of two different strains—CBA and C57BL. In order to create identical conditions for the transplanted cells, the spleen cells were injected intravenously into the hybrid strain, (CBA × C57BL)$F_1$. The animals of the third group (lethally irradiated hybrid recipients) received a cell transplant composed of $10^6$ spleen cells of CBA mice and the same amount of splenocytes of C57BL mice. After seven days, all the animals were sacrificed, and the number of colonies formed by the transplanted cells in their spleens was determined. As we know, the spleen is a lymphoid and haemopoietic organ all in one, in which both lymphocytes and stem cells are present. In our experiment, one of the populations transplanted contained 20, and the other one 15 stem cells. It was reasonably expected that the total number of stem cells in the hybrid strain would be 35 (i.e., 20 + 15), but actually this number appeared to be 12. What had happened to the others? Were they killed or left to the mercy of their

usurper commissioned to prevent them from multiplying and forming colonies according to the formula of 60:30:5?

Assuming it to be the handiwork of lymphocytes, R. Petrov and L. Seslavina carried out a series of experiments that are schematized in Fig. 12. In this case, one group of lethally irradiated recipients was injected with a cell mixture containing stem cells and very few lymphocytes, while the second group animals received a transplant of lymphoid cells. The animals of the third group were injected with

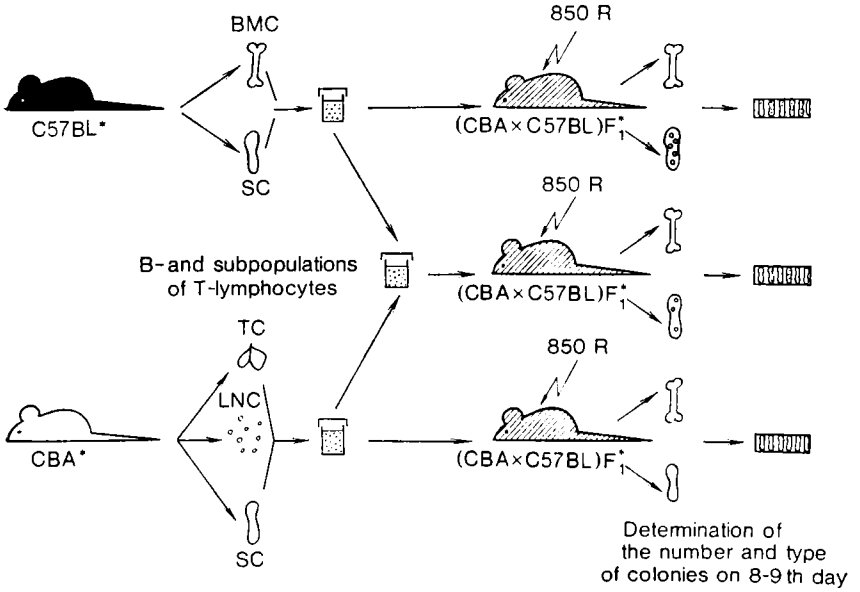



**Fig. 12.** Estimation of inactivating effects of lymphoid cells or their subpopulations on proliferation and maturation of non-syngeneic stem cells. BMC—bone marrow cells; SC—spleen cells; TC—thymus cells; LNC—lymph node cells.

*The choice of donor and recipient genotypes depended on experimental design

a lymphoid-stem cell mixture mimicking the population of spleen cells, with one exception that stem cells and lymphocytes were foreign to each other. Stem cells were obtained from bone marrow cells of C57BL mice; CBA mice were used as donors of lymph node lymphocytes. As in previous studies, lethally irradiated hybrid mice, (CBA × C57BL)$F_1$, served as recipients. The colony growth was revealed only in the spleens of the first group animals immunized with bone marrow cells and was absent in the animals of the second group, to whom lymph node lymphocytes were administered, as well as in the third group animals injected with a mixture of lymphocytes and stem cells. This implied that lymphocytes of CBA mice recognized stem cells of C57BL mice and killed them all, without exception.

This effect has been termed by the authors as "lymphocyte-induced inactivation of non-syngeneic haemopoietic stem cells". "Non-syngeneic" means "non-self", foreign, genetically different as distinct from "syngeneic", i.e. "self", genetically identical. The inactivating effect appeared to be extraordinarily strong. The number of lymphocytes responsible for the recognition of stem cells among the huge variety of other transplanted haemopoietic cells and their subsequent neutralization was 5 times as low as that of the stem cells present in the transplant. Lymphocytes proved to be sharp shooters: the mark was hit perfectly, without a miss.

And again thousands of questions. At this point, Viktor Man'ko takes the floor. Questions posed, questions answered... Attempting to get at the heart of the matter, the authors produce a long series of pro and con statements. Hundreds of experiments are being run in parallel; they are designed to provide evidence for the previously made assumptions and to obtain new, significant results.

**Question.** Can a decrease in the colony number be due to changes in the direction of differentiation of stem cells in response to antigenic stimulation and not to their inactivation by lymphocytes? Lymphocytes of CBA mice are non-syngeneic to haemopoietic precursors of the C57BL strain, aren't they?

**Answer.** Not exactly. In some experiments, lymphocytes were substituted for by renal cells of the same genotype (CBA), or by microbes, both living and dead (Fig. 12). In other studies, we used lymphocytes pre-inactivated by high irradiation doses. In neither case the colony number was diminished. It is thus conceivable that the observed decrease in the number of colonies formed in the spleens of lethally irradiated recipients, to whom a mixture of lymphocytes and non-syngeneic stem cells is administered, is due to inactivation of stem cells by lymphocytes rather than to changes in their maturation. Moreover, haemopoietic precursors can be inactivated only by living lymphocytes; radiation-damaged lymphoid cells are incapable to produce such effects.

**Question.** Does this imply that the changes in stem cell differentiation cannot be the cause of the decrease in the number of visible splenic colonies? Why is this effect induced only by living and not by damaged lymphocytes or other cells, e.g., microbes?

**Answer.** We hold the opinion that the inactivation of stem cells by lymphocytes is the only cause of the decrease in the colony number. By way of illustration, I may cite the following experiment. As in our previous studies, bone marrow cells of C57BL mice were transplanted to lethally irradiated hybrid recipients [(CBA $\times$ $\times$ C57BL)$F_1$], in combination with CBA lymphocytes. In one group, where lymphocytes were in plenty, they killed all stem cells in the graft. In the other group, where lymphocytes were scant, the surviving stem cells could form colonies in the recipients' spleens.

After eight days, the spleens were removed for a visual colony count. Then the spleens were dissected into thin longitudinal cuts (histological slides) and examined under the microscope. In both cases, the results were essentially the same: where lymphocytes were in excess, no colonies could be detected either visually or microscopically. Where lymphocytes were few in number, numerous colonies grew in the spleens, and they could be identified as erythroid, myeloid, etc. Naturally, the microscopic examination allows to reveal more colonies than the visual estimation, since part of the colonies are formed inside the spleen and are inaccessible to visual control. The diminution of the number of every type of colony indicates that the inactivation process involves the precursor cells differentiating via all three branches of haemopoiesis irrespective of the colony type.

**Question.** Is this effect specific for this particular hybrid, i.e., CBA + C57BL → (CBA × C57BL)$F_1$?

**Answer.** Not quite. Evidence of non-syngeneic stem cell inactivation by lymphocytes was obtained in numerous experiments with different mouse genotypes (e.g., A + CBA, A + C57BL, C3H + + C57BL, CBA + (CBA × C57BL)$F_1$, etc.). Furthermore, it is not always necessary to transplant a cell mixture to hybrid recipients. Lymphocytes can inactivate with equal efficiency the stem cells of mice belonging to different genotypes.

**Question.** Is the lymphocyte-induced inactivation of stem cells...

**Table 3.** Inactivation of the Colony-Forming Ability of Stem Cells (C57BL) by Lymphocytes (CBA) in a Cell Mixture Transplanted to Lethally Irradiated Hybrid Recipients, (CBA × C57BL) $F_1$

| Transplanted cells | | | | CBA/C57BL cell ratio | Percentage of damaged stem cells |
|---|---|---|---|---|---|
| CBA mice | Number, $\times 10^6$ | C57BL mice | Number, $\times 10^6$ | | |
| Lymph nodes | 0.2 | Bone marrow | 0.2 | 1 : 1 | 98.9 |
| Ditto | 0.4 | Spleen | 2.0 | 1 : 5 | 93.0 |
| Spleen | 1.0 | Lymph nodes | 1.0 | 1 : 1 | 0 |
| Ditto | 1.0 | Ditto | 10.0 | 1 : 10 | 80.0 |
| Bone marrow | 0.1 | Ditto | 10.0 | 1 : 100 | 99.0 |
| Spleen | 1.0 | Spleen | 1.0 | 1 : 1 | 100* |
| Bone marrow | 0.4 | Bone marrow | 0.4 | 1 : 1 | 36.0 |
| Ditto | 0.05 | Ditto** | 0.05 | 1 : 1 | 77.4* |

* As determined with a T6T6 chromosome marker.
** As determined with bone marrow cells of A mice.

Let us stop for a while the avalanche of questions and look about. Indeed, what do we know about stem cells and lymphocytes?

Lymphocytes interact not only with one another, or with macrophages, but also with haemopoietic stem cells. However, in this case lymphocytes do not stimulate their functional activity as in other cell-to-cell contacts (e.g., lymphocyte-macrophage, T-B, or T-T), but, on the contrary, suppress their ability to proliferate and differentiate into mature blood cells, and thus inactivate stem cells. Lymphocytes destroy stem cells wherever they find them—in the spleen, or in the bone marrow (Table 3). The "hunting" can be unleashed in any locality where living lymphocytes happen to be near at hand (i.e., spleen, bone marrow, lymph nodes—popliteal, subaxillary, submaxillary, mesenterial, etc.).

The manifestation of the inactivating effect does not depend on the time of lymphocyte administration relative to the time of stem cell injection. Lymphocytes that migrate within the organism are always "in order" to counteract the "foreigners": when injected simultaneously with them, 1-4 days prior to stem cell injection, or 1-5 days thereafter. Do you remember our telling you about the devastating war waged by lymphocytes against foreign stem cells? This is exactly the case. Transplanted stem cells migrate within the organism in search of a shelter where they might peacefully proliferate and give rise to lots of new blood cells and thus rescue the organism from the radiation death. Stem cells consider it their predestination, and they do their best to bring it to fulfilment. However, every now and then, the migrating stem cells run against the highly labile frontier-guards that themselves roam through nooks and corners of the body, seeking for foreign cells. The stem cells of the transplant are foreign to the lymphocytes of another genotype, and for "foreigners" lymphocytes "feel" no pity: they kill them at the very first encounter. This process is not species-specific and involves cells of different genotypes.

Thus, lymphocytes inactivate haemopoietic stem cells that are foreign to them. But do they really? Another benefit of doubt—and a new series of experiments...

### In Search of Labelled Chromosomes

As we have seen in preceding sections, haemopoietic stem cells differentiate in three main directions and ultimately evolve into various blood cells (erythrocytes, leucocytes, thrombocytes) which, in turn, via a number of intermediate forms, give rise to T- and B-lymphocytes. T- and B-cells that further develop on their own are discriminated by the type of immune reactions mediated by them, i.e., cell-type and humoral immune responses. What makes stem cells differentiate in one particular direction,

e.g., into T-lymphocytes, in an adult organism? Are they impelled to do so by lymphocytes? Indeed, in order to raise the power of the army of immunity, one has to recruit increasing numbers of lymphoid cells. If this is true, then the absence of colony growth as a result of stem cell inactivation may not necessarily be due to the destruction of haemopoietic precursors. At the command from lymphocytes, stem cells are prone to change their lifecycle and further differentiate into lymphoid, instead of blood, cells. Is that the reason why they cannot be detected visually or microscopically? For until very recently, investigators had been seeking for colonies of haemopoietic, but not lymphoid, cells.

Attempting to resolve this problem, R. Khaitov from our group carried out the following experiment. A mixture of bone marrow cells of mice belonging to two different genotypes was transplanted to lethally irradiated recipients. Such experimental systems have two major implications, both theoretical and practical. The bone marrow carries a certain amount of lymphocytes; their number, though, is much fewer than that in the spleen or lymph nodes. But what if the bone marrow transplant contains cells obtained from two different donors? Will these cells kill one another? Earlier, we demonstrated that a decrease in the colony growth is observed both upon transplantation of a bone marrow—lymphocyte mixture to lethally irradiated recipients and upon administration of bone marrow cells obtained from different murine genomes. Is it inactivation, then? This problem is of particular importance because transplantations of bone marrow cells obtained from several donors is the usual practice in the therapy of some forms of anemia, leukemia, and radiation disease. The point is that in the mass the bone marrow cells of the transplant are foreign to the organism, but one of the donor samples may appear particularly well-suited for the recipient. Therefore, the greater the number of potential donors, the higher the chances of adaptation of a certain component of the mixed transplant. However, if the stem cells in the mixture have been mutually destroyed, the transplantation whose sole purpose is to make stem cells adapt and multiply in the damaged organism becomes meaningless.

We conducted a series of experiments with bone marrow cells labelled with the T6T6 chromosome marker. After administration to lethally irradiated recipients of bone marrow cells from one donor, all of the tissues under study (i.e., spleen, thymus, lymph nodes, bone marrow) appeared to contain the label, which is a sign of good proliferation of stem cells within the radiation-damaged recipient's organism. However, in the tissues of recipients to whom a mixture of bone marrow cells from two different donors was transplanted, the label was absent. In this case, all of the stem cells of the second genotype had been killed by the lymphocytes present in the bone

marrow transplant. The same was observed when bone marrow cells mixed with non-syngeneic lymphocytes were administered to lethally irradiated recipients.

Thus, the answer to the question posed is quite unambiguous: bone marrow cells to be transplanted to an alien organism should not be obtained from two or more donors, otherwise the haemopoietic stem cells of the transplant will kill one another.

These experiments clearly demonstrated that lymphocytes do not change the direction of differentiation of non-syngeneic stem cells, they just kill them. But how do they do it? By coming into contact

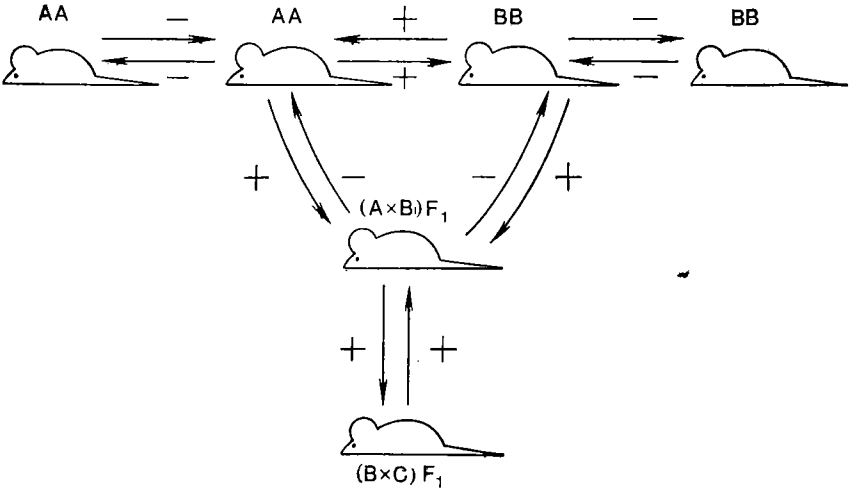



**Fig. 13.** Direction of immunologic reactions during transplantation of lymphoid cells between mice of different genotypes (AA, BB, and CC). + response; — lack of response

with them or indirectly, within the organism of a recipient to whom a mixture of non-syngeneic cells is being transplanted? In most cases, not only haemopoietic stem cells but also the whole organism of the recipient is non-syngeneic to the lymphocytes. It is therefore not unnatural that lymphocytes immediately attack the foreign tissues and alter them to such an extent that they can no longer serve as a shelter for stem cells, and the latter die without even having a start in life.

Our subsequent experiments were performed in conformity with immunogenetic laws of histocompatibility upon transplantation. A typical experiment with transplantation of lymphoid cells between related and unrelated species and their first generation hybrids is schematized in Fig. 13.

Cell transfusions from AA to AA and from BB to BB mouse strains do not evoke immune responses. These cell lines are geneti-

cally identical, there is no antigenic antagonism between them. Nor are tissue incompatibility responses evoked, when $(A \times B)F_1$ hybrid cells are administered to AA or BB strains. The tissues of parental strains contain no antigenic determinants against hybrid cells that are absent in their own genome. In all other combinations (Fig. 12), lymphocytes trigger off immune responses against the antigenic material that is absent in their genomes. Actually, immune responses are evoked when AA lymphocytes are transplanted to BB mice, or when BB lymphocytes are administered to AA mice. If AA cells are transplanted to $(A \times B)F_1$ hybrids, AA lymphocytes are challenged by antigens B present in the $(A \times B)F_1$ genome. Conversely, when BB cells are administered to mice of the hybrid strain, incompatibility responses are evoked; in this case BB lymphocytes are challenged by antigenic molecules A present in the $(A \times B)F_1$ genome. By analogy, lymphocytes of $(A \times B)F_1$ hybrids yield incompatibility responses against antigens C present in the $(B \times C)F_1$ genome, while those of the $(B \times C)F_1$ strain evoke similar immune responses against antigen A present in the $(A \times B)F_1$ genome.

With this in mind, R. Khaitov carried out the following experiment. Lethally irradiated recipients (CBA) bearing the T6T6 chromosome marker (CBAT6T6) received a transplant of spleen cells of the hybrid strains, $(CBA \times C57BL)F_1$ and $(A \times CBAT6T6)F_1$. According to transplantation laws, neither of the lymphocytes of the hybrid strains could evoke incompatibility responses against the antigens of CBA whose genome contains a similar repertoire of antigenic determinants. At the same time, for the lymphocytes of $(A \times CBAT6T6)F_1$, the antigenic molecules of C57BL in the $(CBA \times C57BL)F_1$ genome are non-syngeneic; likewise, the antigenic molecules of the A strain present in the $(A \times CBAT6T6)F_1$ genome are non-syngeneic to the $(CBA \times C57BL)F_1$ hybrids. The lymphocytes of the parental genotype (CBAT6T6), that are irreversibly damaged by lethal irradiation cannot evoke incompatibility responses against the transplanted hybrid cells. In this version of experiment, lymphocytes of hybrid mice could reciprocally inactivate their own stem cells, for every possibility of their interaction with the stem cells of the recipient (CBAT6T6) and vice versa was excluded.

It is of interest that all the three genotypes used in this study contained different amounts of labelled chromosomes: CBAT6T6 had two, $(A \times CBAT6T6)F_1$ had one, while $(CBA \times C57BL)F_1$ had none! This finding made it possible to establish, with a high degree of accuracy, which of the proliferating cells were inactivated, and when and where the inactivation took place. The changes in bone marrow, thymus, spleen and lymph node lymphocytes of CBAT6T6 recipients were followed over a period of 30 days. Although any rejection of the cell graft by the recipient's organism as measured by the "graft-versus-host" (GVH) response was theoretically impos-

sible, the inactivation did take place. None of the tissues under study appeared to contain proliferating cells of the (CBA × C57BL)$F_1$ genotype. Accordingly, the inactivation of non-syngeneic stem cells by lymphocytes is a comprehensive process which is not mediated by the recipient's organism and is independent of the GVH response.

In the experiments described above, the "killer" parts were performed by (A × CBAT6T6)$F_1$ lymphocytes; the stem cells of the (CBA × C57BL)$F_1$ strain served as "targets". However, the subdivision into "killers" and "targets" is arbitrary and is not an intrinsic, everlasting property of a particular genotype conferred to it by nature. In different genetic combinations, the cells of the same genotype can act both as killers and targets depending on the situation. For instance, in the combination of CBA and C57BL strains, lymphocytes of CBA mice function as killers, while stem cells of C57BL mice as targets. When spleen cells of A and CBA mice are administered to lethally irradiated recipients, stem cells of the CBA strain act as targets, while those of the A strain as killers. At the cell ratio of 1 : 1, the reaction is always unidirectional and it terminates in complete destruction of stem cells of the inactivated genotype. The unidirectionality is a temporary event, though. Intravenous injections of lethally irradiated recipients with $10^6$ lymph node lymphocytes of C57BL mice and $10^6$ spleen cells of CBA mice do not result in stem cell inactivation: in this case, lymphocytes of C57BL do not reveal their killing properties. However, an increase in the number of lymphocytes of C57BL by one order of magnitude, i.e. totalling 10,000,000, is accompanied by marked inactivation of haemopoietic stem cells of the CBA strain. In this instance, lymphocytes kill about 80% of non-syngeneic stem cells (Table 3).

The degree of inactivation depends decisively on the genotype of the recipient, to whom a mixture of lymphocytes and non-syngeneic stem cells is administered. We transplanted the same cell mixture to lethally irradiated mice belonging to different genotypes. In these experiments, lymphocytes of CBA caused a practically complete destruction of stem cells of C57BL in the (CBA × C57BL)$F_1$ (2 : 1), CBA (10 : 1), and C57BL (200 : 1) murine strains.

The occurrence and rate of stem cell inactivation by lymphocytes depend on a number of factors, such as the genotypes of the interacting species and their combinations, ratios of interacting cells as well as the genotype of the recipient of a mixed cell transplant. Obviously, the crucial role in the interactions between the key cells of the immune and haemopoietic systems belongs to genetic aspects.

Our story would be incomplete without mention of another line of our research. Spleen cells that are usually transplanted to lethally irradiated recipients contain both stem cells and lymphocytes. As we have seen in the foregoing experiments, the inactivation process is not mediated by the recipient's organism. But can this

process be influenced by the interactions between donor lymphocytes of the two genotypes used in the experiment? Attempting to solve this problem, R. Khaitov immunized lethally irradiated recipients (CBA) not with spleen cells of two hybrid genotypes but with their lymph node lymphocytes, to which bone marrow cells of CBA mice (i.e., those that are capable of forming colonies in the recipients' spleens) were added. It was expected that if the inactivation was mediated by the interaction between lymphocytes of these two genotypes, stem cells would be unable to form colonies within the recipient's organism because of their inability to proliferate. It appeared that the transplanted bone marrow cells of CBA mice formed 15 colonies in the recipients' spleens, which indicated that lymphocytes of the hybrid strains, (CBA $\times$ C57BL)$F_1$ and (A $\times$ CBA)$F_1$ transplanted simultaneously with them had failed to inactivate their stem cells. According to trasplantation laws, lymphocytes could not yield incompatibility responses against those stem cells; therefore, the number of colonies formed by stem cells in the spleens of CBA recipients was practically the same as that grown after administration of bone marrow cells of CBA mice along, i.e., 16.1 and 15.3, respectively. In the third group animals immunized with a mixture of lymphocytes of the (CBA $\times$ C57BL)$F_1$ and (A $\times$ CBA)$F_1$ hybrid strains and bone marrow cells of the CBA strain, the average number of newly formed colonies was 14.5. As we know, lymphocytes of (CBA $\times$ C57BL)$F_1$ mice can develop incompatibility responses against bone marrow cells of the (A $\times$ CBA)$F_1$ strain, which, in turn, evoke cell-type immune responses against (CBA $\times$ C57BL)$F_1$ cell transplants. And yet, despite the reciprocal hostilities, the lymphoid cells of these genotypes failed to inactivate stem cells which could form colonies in much the same way as in the absence of lymphocytes.

Hence, the inactivation of non-syngeneic stem cells by lymphocytes does not involve any additional cells of a recipient or donor origin. Such interactions are hardly possible because of the ability of lymphocytes to secrete inhibiting factors. Lymphocytes interact with and inactivate non-syngeneic stem cells directly by virtue of special surface macromolecules, or receptors.

### What Happens Within One Hour?

One of the most urgent problems demanding immediate solution is the continuance of the stem cell destruction process. To know it exactly is very important, because timely measures must be taken to rescue stem cells and eliminate the killing activity of lymphocytes. These efforts will be fruitless, however, if the help arrives too late, when lymphocytes have committed their reprisal over stem cells. The latter can be saved only when they are still

alive. Usually, in order to see the result of lymphocyte—stem cell interaction, 8 or 10 days after transplantation, the spleens of lethally irradiated recipients are removed for a colony count. This is the time needed for stem cells to proliferate in sufficient amounts and to form visible colonies; no colonies can be visualized before the time. But if the colony growth is absent, how should one know when stem cells were destroyed by lymphocytes, within one or twenty-four hours after administration of the mixed transplant? Can this process take a still longer course? What are the events preceding the inactivation?

In an attempt to answer these questions, we conducted a series of experiments with an antilymphocytic serum (ALS), a remarkable preparation which is highly active towards lymphoid cells, especially circulating $T_2$-lymphocytes. ALS was injected to lethally irradiated mice immediately after administration of a lymphocyte—non-syngeneic stem cell mixture. Under these conditions, ALS killed all lymphocytes and thus eliminated their inhibiting action upon the stem elements; as a result, all of the stem cells survived and formed numerous colonies in the recipients' spleens. However, when ALS was injected one hour after the transplantation of the cell mixture, no protection of the stem cells was afforded: by that time lymphocytes had parried the main blow and made stem cells infertile.

What happened within one hour? Were all stem cells destroyed or did most of the lymphocytes occupy the loci inhabited by stem cells and thus became inaccessible for ALS? If that were true, lymphocytes would keep on killing stem cells and even ALS would be unable to stop this hazardous process. Therefore, the experiments should be designed so that the fate of stem cells, dead or alive, is always in the focus of attention. One must always be ready to locate lymphocytes in the areas where stem cells form their colonies, i.e., in the spleen and bone marrow. Such an experimental system has been developed (Fig. 14) and it is based on the following principles.

As we have seen, intravenous administration of a lymphocyte—non-syngeneic bone marrow cell mixture to lethally irradiated recipients leads to the inactivation of stem cells of the transplant as well as to the loss of their colony-forming ability. Naturally, spleen cells obtained from such animals and transplanted to other lethally irradiated recipients will be unable to form colonies, being inactivated by lymphocytes. However, if stem cells are alive or destroyed only partly, transplantation of spleen cells from one lethally irradiated recipient to another will permit to determine the number of surviving stem cells from the number of colonies formed by them. Changing the duration of non-syngeneic cell contacts within the organism of primary recipients, one can determine the time necessary for the full inactivation of haemopoietic precur-

sors to take place. Retransplantation of various tissues (e.g., spleen, bone marrow) makes it possible to measure the rates of stem cell inactivation in different parts of the organism. And, finally, the inactivating effect of lymphocytes present in the retransplanted tissue can be abolished by ALS administered to some of the secondary recipients simultaneously with the transplant. Any increase in the colony number is a sign that lymphocytes are present in the retransplanted tissue and they inactivate the stem cells of the

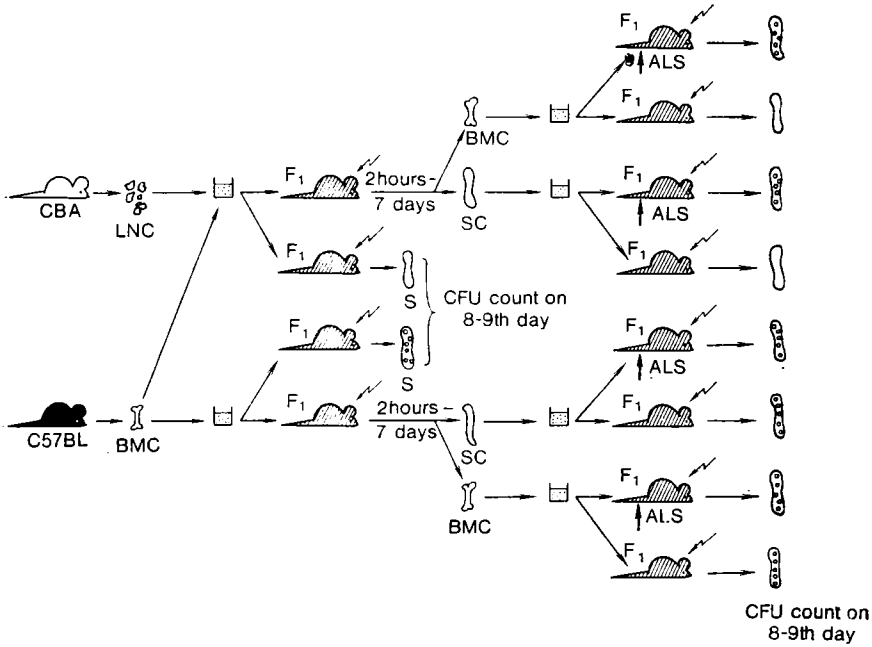



**Fig. 14.** Determination of the time necessary for inactivation of non-syngeneic stem cells by lymphocytes in the spleen and bone marrow of lethally irradiated recipients.

LNC—lymph node cells; BMC—bone marrow cells; SC—spleen cells; CFU—colony-forming units; ALS—antilymphocytic serum; $F_1$—lethally irradiated mice, (CBA × C57BL)$F_1$; S—spleen

secondary recipients. The lack of colony growth under ALS influence suggests that the retransplanted tissue contains no lymphocytes.

These experiments were carried out in two series. In one, spleen and bone marrow cells of lethally irradiated mice were retransplanted 2 hours and 1, 3, 5, and 7 days after immunization of the animals with bone marrow cells without lymphocytes. In the other, haemopoietic cells were retransplanted at the same time intervals to lethally irradiated recipients pre-immunized with a mixture of lymphocytes and non-syngeneic stem cells. Eight days after cell

retransplantation and ALS injection, the animals were sacrificed, and their spleens were removed for a colony count. Comparing the number of splenic colonies in both experimental groups, we could determine the degree of stem cell inactivation at different periods of time after transplantation of a non-syngeneic cell mixture to the recipients.

The results of these experiments present substantial interest. Haemopoietic stem cells appear in the spleens and bone marrow of the recipients already within the first 2 hours after transplantation of the non-syngeneic cell mixture. The stem elements are delivered

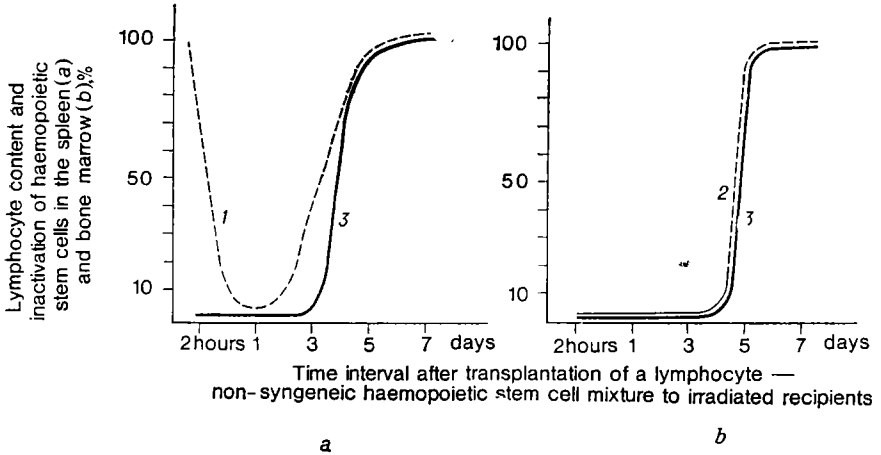



**Fig. 15.** Determination of the number of lymphocytes and the time of inactivation by lymphocytes of non-syngeneic stem cells in the spleen (*a*) and lymph nodes (*b*) of lethally irradiated recipients:
*1*—percentage of lymphocytes in the spleen; *2*—percentage of lymphocytes in the bone marrow; *3*—inactivation of non-syngeneic stem cells.

to these organs by the circulating blood, and there they start to proliferate and maturate into blood cells necessary to protect the damaged organism from the radiation death. Nor do the lymphocytes procrastinate: they are also conveyed to the spleen with the blood and there happen upon the multitudes of stem cells ready to begin their life cycle. Urgent measures ought to be taken by the lymphocytes—to increase in number and block the way for migrating stem cells. Once the lymphocytes have recognized and located the enemy, they leave the spleen. Twenty-four hours after the transplantation, there is not a lymphocyte to be seen in the spleen or bone marrow (Fig. 15). As for the stem cells, none of them was destroyed by retransplantation: these cells survived and formed colonies in the secondary recipients. Meanwhile, the lymphocytes

migrate to the lymph nodes. There, they proliferate and increase in number, reinforce their troops, forewarn their "brothers-in-arms" about the threatening danger, and post sentries. Having taken measures of precaution, the lymphocytes return to the spleen and bone marrow occupied by "foreign" stem cells. Their advent signifies death for the stem cells; their decay lasts 3 (in the spleen) or 4 (in the bone marrow) days, and in a day or two all non-syngeneic precursor cells appear to be dead.

This horrifying account is partly a product of our fantasy. In fact, we do not know in what direction lymphocytes migrate from the spleen and what happens to them thereafter. In every other detail, this picture mirrors the authentic events that are shown graphically in Fig. 15. Inactivation of non-syngeneic stem cells by lymphocytes is a multistep process which follows a certain time course. Initially, transplanted cells interact with one another and rapidly "colonize" the irradiated recipient's tissues. This process normally takes a few hours. During the next 3-4 days, lymphocytes are redistributed within the recipient and are predominantly accumulated in the spleen and bone marrow. At this time, they begin to inactivate the non-syngeneic stem elements. The inactivation is completed within 1-2 days, i.e., on the 4-5th day after transplantation. There is some evidence that the last steps of this process are mediated by sensitized lymphocytes challenged by antigenic macromolecules that are common for stem cells and the recipient.

The above-described experiments were performed in lethally irradiated mice of CBA and C57BL as lymphocyte and bone marrow donors, respectively; (CBA $\times$ C57BL)$F_1$ mice were used as primary and secondary recipients.

## Lymphocytes, Stem Cells, and Chemotherapy

On the 5th post-transplantation day, lymphocytes win a brisk, triumphant victory over stem cells—the latter are all dead. This success, however, would have been complete, if lymphocytes had destroyed their own mutant cells that threaten with malignancy. In our instance, when stem cells eager to save the organism from radiation death themselves fell victims to lymphocytes, the victory is rather a defeat for the whole organism, and for lymphocytes, in the first place. In order to let stem cells survive, proliferate, and save the radiation-damaged tissues, the destructive activity of lymphocytes has to be arrested at any price. The deliverance may come from special lymphotropic drugs that would selectively block the activity of lymphocytes without affecting that of stem cells. Such compounds have to be sought among the already known ones or be created anew. On the look-out for lymphotropic drugs, the above-described experimental system based on inactivation of haemopoietic

stem cells by lymphocytes seems to be a convenient and perspective one.

Bone marrow stem cells intravenously administered to lethally irradiated recipients form colonies in their spleens. No such colonies are formed, however, when stem cells are transplanted simultaneously with non-syngeneic lymphocytes: the latter inactivate all stem elements in the graft. Within the recipient's organism, two antagonistic processes occur in parallel: stem cells tend to proliferate and form colonies, while lymphocytes do their best to destroy these cells because they are foreign to them. Lymphocytes win—and no colonies are formed. But what if a third component (e.g., a previously known or a newly synthesized compound) is added to this system? If the added substance is neutral in effect or poisonous with respect to lymphocytes and stem cells, no colonies will be formed. The result will be the same, if this substance is toxic only for haemopoietic cells. But if the third component in the mixture is capable of selectively blocking the activity of lymphocytes without doing any harm to stem cells, the latter will retain their colony-forming ability. In 1969, a group of Soviet immunologists (R. Petrov, V. Man'ko, R. Khaitov, and L. Seslavina) elaborated a simple practicable approach to a selection of immunodepressants and lymphotropic drugs. The clinical applicability of these agents is not delimited by their use in the therapy of radiation sickness by bone marrow cell transfusions. Lymphotoxic agents which possess the ability to selectively block the activity of lymphocytes have proved to be a useful tool in the suppression of various immune responses during organ transplantations or in the therapy of autoimmune diseases, some forms of leukemia, and other disorders. These preparations are not supposed to exert a general mitostatic effect, i.e., they should not suppress cell proliferation, especially that of haemopoietic precursors. Such a deliberate, exacting approach to selection of curative agents is justifiable, since most of the currently known immunodepressants exhibit a strong mitostatic activity and destroy many rapidly proliferating tissues, such as bone marrow, intestinal epithelium, skin, etc.

A schematic representation of a test system for selection of lymphotoxic agents is given in Fig. 16. The lymphotoxic properties of a particular compound are estimated in lethally irradiated mice pre-immunized with a mixture of lymphocytes and non-syngeneic stem cells. If the compound tested is able to alleviate the inactivation effect of lymphocytes on stem elements, it is said to be lymphotoxic; the absence of colony growth suggests that its lymphotoxicity is zero. The usual practice in the estimation of the mitostatic effect of a given compound is its administration to lethally irradiated recipients pre-immunized with bone marrow cells. The appearance of splenic colonies is a sign that the preparation in a given dose

has no mitostatic effect and, vice versa, the absence of colony growth points to a strong mitostatic activity of this drug.

This test system has been used for the analysis of an immense variety of substances taken at different doses. The compounds tested revealed a broad spectrum of theoretically expected specificity. The antilymphocytic serum ALS) appeared to have a very wide

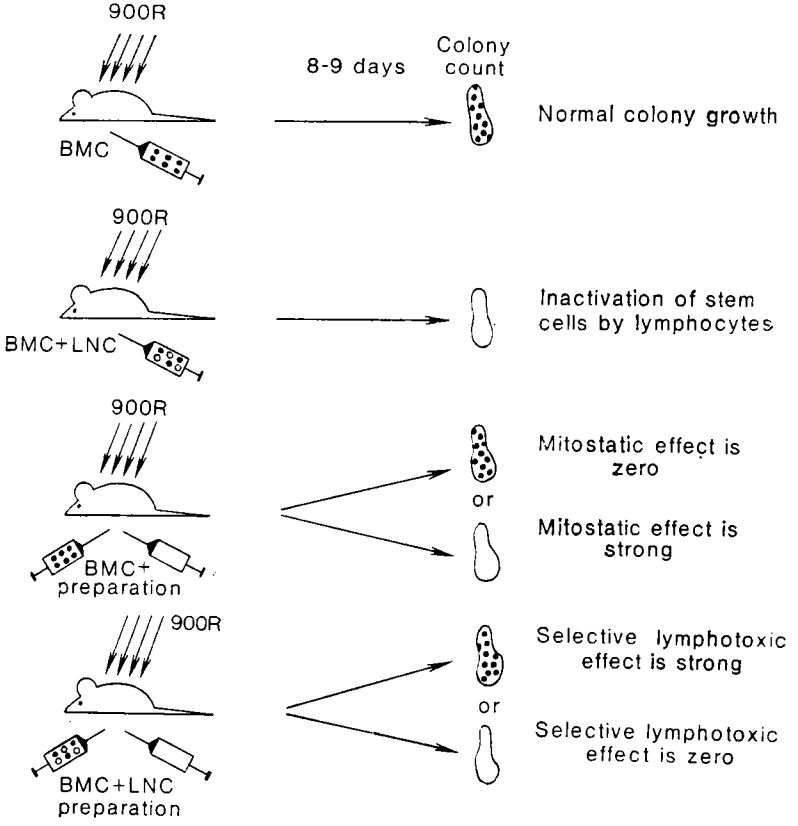



**Fig. 16. Determination of lymphotoxic and mitostatic properties of various compounds.**
BMC—bone marrow cells; LNC—lymph node cells

selective lymphotoxicity, while cyclophosphamide and hydrocortisone acetate had a much narrower range of specificity. 6-Mercaptopurine and imurane were fairly inefficient and exerted, in addition, strong mitostatic effects. Our test system was successfully employed by investigators from many laboratories.

The benefits of the proposed method of selective blocking of lymphocytes have provided an entirely new approach to the problem

of medicinal control over immunity. In 1971, R. Petrov and V. Man'ko formulated novel principles in immunodepression which, in a concise form, can be presented as follows. Immunogenesis, the ultimate stage of cell conversions terminating in the production of sensitized lymphocytes or antibody-secreting cells, is preceded by a number of intermediate steps. The most important of them is the interaction between lymphocytes and other cells of the body, e.g., haemopoietic stem precursors, other lymphocyte types, and macrophages. The new principles of control over the "mechanism of immunity" would implicate a search for ways and means of regulating various cell interactions, i.e., their stimulation or blocking (depression). Such an approach is also oriented towards establishing directional control over individual links in the immunogenetic process. To our minds, an "ideal" immunodepressant for the regulation of humoral immune responses is the one that exerts a pronounced selective lymphotoxic effect and suppresses, in addition, T-B cell interactions and immunocompetent cell migration. Conversely, an "ideal" immunostimulator is the one which stimulates T-B interactions and immunocompetent cell migration but does not show lymphotoxic effects. Neither of them should display mitostatic activity.

**Table 4.** Properties of "Ideal" Immunodepressants and Immunostimulators

| Preparation | T-B interactions | | | Migration of cells | | Cytotoxicity |
|---|---|---|---|---|---|---|
| | Suppression | Stimulation | Suppression | Stimulation | Mitostatic activity | Lymphotoxicity |
| Immunodepressant | + | − | + | − | − | + |
| Immunostimulator | − | + | − | + | − | − |

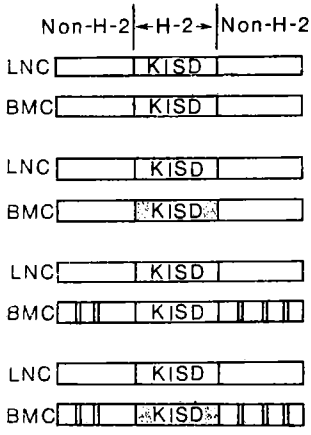
Needless to say that the "ideal" properties of an immunoregulatory drug have to be specified in each individual case. By way of example, we may cite another extract from the book by E. Gruntenko "Immunity: Pros and Cons".

It is well known that certain malignancies are concomitant with activation of T- and B-suppressors and inhibition (or lack) of antitumour immune responses. Total stimulation of the immune system may considerably aggravate the situation, resulting in amplification of the suppressor activity of lymphocytes and further suppression of antitumour defense systems. To this end, selective blocking of excessive activity of suppressor lymphocytes and selective activation of lymphoid cell subpopulations responsible for the induction of antitumour immunity seem to be far more promising approaches.

## Genes, Lymphocytes, and Stem Cells

Inactivation of non-syngeneic stem cells by lymphocytes is one of manifestations of tissue incompatibility which, in contrast with other known incompatibility responses, proceeds exceedingly fast. The rejection of a skin graft, for instance, takes 12 to 14 days and involves the most essential components of the damaged tissue, i.e., the cells responsible for tissue regeneration. The transplanted tissue is still alive, but if its basic elements—stem cells—are destroyed, the whole graft is doomed to death.

What genetic systems control this type of tissue incompatibility? In the USSR, this problem has been extensively studied by Eduard




| Genotypes of BMC and LNC donors | LNC:BMC | Inactivation,% |
|---|---|---|
| CBA+CBA, C57BL+C57BL | 1 : 1 | 0 |
| | 5 : 1 | 0 |
| A/Sn+A.CA, A.CA+A/Sn, B10.BR+B10.CNB, B10.CNB+B10.BR, B10.BR+B10, B10+B10.BR | 1 : 1 | 0 |
| | 5 : 1 | 43 |
| AKR+C3H, AKR+CBA, CBA+C3H, BALB/c+DBA/2, B10.BR+CBA, B10.BR+AKR | 1 : 1 | 62 |
| | 5 : 1 | 65 |
| CBA+C57BL, AKR+DBA/2, C3H+C57BL, A/Sn+C57BL, A/Sn+CBA, DBA/2+C57BL | 1 : 1 | 100 |

**Fig. 17.** Inactivation of non-syngeneic stem cells by lymphocytes in different genetic combinations of bone marrow and lymphocyte donors (hatched area) in H-2 and non-H-2 systems.

BMC—bone marrow cells; LNC—lymph node cells

Panteleev and Ilariah Dishkant. All previously established incompatibility responses were said to be due to incongruities in the so-called major histocompatibility complex, i.e., the HLA system in man and H-2 system in mice. The murine H-2 system is localized in the seventeenth chromosome and comprises four main regions: K, I, S, and D. If the donor's genes encoded by the corresponding regions differ from those of the recipient, strong incompatibility responses may be evoked. As we know already, skin grafts are rejected within 12-14 days. If all the genes in a given system are identical, and occasional mutations do not influence H-2 genes, incompatibility responses are weak, and the skin may be rejected during several months. This has been the general rule for every type of the so far known incompatibility responses. However, the inactivating effect

of lymphocytes on stem cells is an exception from this rule. Genetic differences irrelevant to the H-2 system appear to be far more significant than H-2 genes (Fig. 17). Hundreds of experiments have been carried out in our laboratory over the last decade, and those involved an immensely large number of genetic combinations of lymphocytes and stem cells. The results of these experiments were reported at the International Congress on Transplantology in New York in 1976. The main idea of this presentation was that stem cell inactivation by lymphocytes is controlled by a genetic system other that H-2. This spectacular finding was acknowledged as an entirely new phenomenon.

Lymphocytes have the ability to disclose, recognize and kill stem cells obtained from mutant (genetically transformed) animal strains that differ from them only in one mutant gene. This implies that lymphocyte-induced inactivation of non-syngeneic stem cell is related to the primary function of the immune system, namely, the immunologic control providing for day-by-day protection against cancer. Out of $10^{13}$ cells of the human body, $10^6$-$10^7$ cells are subjected to occasional mutations, i.e., at any moment no less than a million mutant cells including cancer ones, circulate in the organism. Someone must uncover and destroy them. The executor's function fell to the share of lymphocytes.

The prominent role of lymphoid cells standing guard over the genetic equilibrium of the organism can be best illustrated by their ability to inactivate various non-syngeneic precursor cells involved in vigorous proliferation and differentiation. Studies in this field were initiated in 1967 by R. Petrov, E. Panteleev, and L. Seslavina who investigated miscellaneous effects of lymphoid cells on non-syngeneic precursors of antibody-producing cells. As in the foregoing experiments, lethally irradiated recipients were immunized with spleen cells mixed with antigens (sheep erythrocytes). As a result, numerous cells capable of making antibodies against sheep erythrocytes began to accumulate in the recipients' spleens. After addition of a third component (e.g., spleen cells of another genotype) to this experimental system, the number of antibody-producing cells decreased sharply: the lymphocytes present in the transplant had neutralized their precursors.

Besides, we studied various interactions of lymphocytes with non-syngeneic precursors of antibody producers during primary and secondary immune responses, using spleen cells challenged with sheep erythrocytes. When very thin sections of mouse spleen tissue were placed onto agar dishes filled with a suspension of sheep erythrocytes (complement), the antibodies secreted by a cell colony of antibody-producing cells brought the underlying erythrocytes to lysis. Since every colony is an independent clone of cells produced by the same precursor, the number of lytic zones exactly corresponds

to the number of active antibody producer precursors in the spleen.

Using this technique, we obtained compelling evidence in favour of the lymphocyte-induced inactivation of non-syngeneic precursors of antibody-producing cells. The diminution of the number of these precursors and the corresponding decrease in the number of antibody-secreting cells produced by them are observed both during the primary and secondary immune responses to erythrocytic antigens. During the secondary immune response, antibody producer precursors are inactivated to a much greater extent due to a more vigorous proliferation of these cells. Such an intensive division of foreign elements is an alarm signal for lymphocytes to launch an attack, for they are particularly hostile to cells with a high reproductive power.

Evidence for this assumption may be derived from the following experiments. Administration of a cell mixture composed of an antigen (sheep erythrocytes), lymph node lymphocytes of CBA mice and non-syngeneic spleen cells of the C57BL strain to irradiated recipients results in the inactivation of antibody producer precursors by lymphocytes: the spleen cells of these animals contain no antibody-producing cells. However, when lymphocytes are injected to the same recipients 1-3 days after the administration of the spleen cell-antigen mixture, i.e., after the first "cell population explosion", the number of antibody-producing cells increases 3-4-fold. In this case, lymphocytes do not inactivate non-syngeneic cells; instead, they interact with them, as a result of which the number of antibody producers in the recipients' spleens markedly increases.

The observed stimulation of antibody genesis during the primary immune response is due to cooperative interactions between the immunizing lymphocytes and the antigen-challenged non-syngeneic stem cells. In the productive phase of antibody synthesis, these cell interactions may involve genetically irrelevant cells. There is no cooperativity, however, between genetically different cells during the secondary immune response. A more detailed description of intercellular events occurring at the level of mature antibody producers is given in the chapter "Molecular Messengers of Immunity".

In experiments by E. Panteleev (USSR), spleen cells of parental genotypes were transplanted to intact hybrid animals and their "attacking" activity toward the recipients' tissues was investigated. In accordance with transplantation laws (Fig. 13), in the given combination hybrid recipients cannot evoke immunologic responses against the antigenic material of the transplant. Only parental cells can do it, since the latter contains antigenic determinants that are absent in their genome. The GVH response appeared to be much less pronounced in animals immunized with a mixture of non-syngeneic lymphocytes: in this case, too, lymphoid cells of one genotype killed precursor cells of another genotype.

When studying the interactions between lymphocytes and non-syngeneic stem cells, we found that the inactivation process is not confined to the spleen but may take place in other parts of the organism as well. Under the microscope, stained histological slides of the femoral bone of irradiated recipients immunized with bone marrow cells, reveal distinct haemopoietic foci formed by the transplanted stem cells. No such foci can be visualized, when a mixture of non-syngeneic stem cells and lymphocytes is administered to these recipients, because all of the stem cells occupying the bone marrow cavity are killed by the lymphocytes. The colony growth is zero. The same fate awaits the non-syngeneic precursors of osteoblasts: lymphocytes do not spare them either. Femur cuts of irradiated recipients immunized with a lymphocyte—non-syngeneic bone marrow cell mixture reveal no traces of osteoblasts, i.e., the elements that further evolve into bone cells, or osteocytes.

These examples provide demonstrative evidence of strong inactivating effects of lymphocytes on non-syngeneic haemopoietic precursors that further proliferate and differentiate into mature blood cells and fully agree with the hypothesis put forward by R. Petrov as long ago as 1968. According to this hypothesis, lymphocytes can inactivate many types of precursor cells which, similar to stem cells, have the ability to proliferate and differentiate into mature cells. Conceivably, different methods have to be used for evaluating this inactivating effect, e.g., selective suppression of functional activities of certain cell elements, or subtraction of definite cell populations from a cell mixture, etc.

It is becoming increasingly evident that interactions of non-syngeneic stem cells with lymphocytes and their subsequent inactivation is a universal process which can be directed against any proliferating or differentiating cell population irrespective of its localization in the haemopoietic system or in other parts of the organism. This conclusion may be exemplified by experiments of Nataliya Kompaniets (USSR), who reproduced the methodology pioneered by M. Seki (Japan), i.e., cultivation of bone marrow cells in the peritoneal cavity of lethally irradiated mice. If a very thin membrane perforated with tiny holes that are penetrable for solute nutrients but impenetrable for cells is placed into the peritoneal cavity of mice, in 7 days it will be densely covered with peritoneal cells, for the most part, macrophages. The animals carrying such intraperitoneal membranes are irradiated with a lethal dose and injected with bone marrow cells which form haemopoietic foci on the surface of the macrophages. The colonies formed are predominantly of a myeloid type; their number can be easily determined under the microscope. For a microscopic assay, the colonies are carefully transferred to specially stained films placed on top of glass plates (slides). A very interesting peculiarity of the colonies formed by bone marrow cells in the

peritoneal cavity of lethally irradiated recipients is that their growth is not influenced either by the haemopoietic system (the membrane is not "germinated" by blood vessels during the cultivation) or by the lymphoid systems "architecturally" moulded into lymphoid and haemopoietic organs. N. Kompaniets extended this procedure by including into the system a mixture of lymphocytes and non-syngeneic bone marrow cells; as a result, not a colony was formed. In the absence of lymphocytes, stem cell precursors could proliferate, differentiate, and form haemopoietic foci, but not when lymphocytes were present. So, in this case, too, non-syngeneic precursor cells appeared to be killed by lymphocytes.

In such way, lymphocytes, endeavouring to save the organism from the harmful influence of their own mutants or foreign stem cells, destroy all genetically unrelated elements taking the path of active proliferation and differentiation. Even one mutant gene can induce lymphocytes to launch an attack terminating in its destruction. As we have seen, the inactivation of non-syngeneic stem cells by lymphocytes is a relatively fast process that is normally completed within 3-5 days, and it may be evoked in any part of the organism, even beyond the bounds of the circulatory and lymphoid systems.

**Properties of Inactivators**

Information regarding the type of lymphocyte subpopulations capable of interacting with non-syngeneic haemopoietic precursors and the properties of lymphoid cells responsible for stem cell destruction, provides guidelines for future studies on interactions between the central cells of haemopoietic and immune systems. This problem arose from the very outset, and in all subsequent inactivation experiments our main attention was focussed on the cells that initiate this process, i.e., lymphocytes. An estimable contribution to the study of inactivator lymphocytes has been made, besides the authors of this book, by Galina Ignatyeva, Nataliya Khalatyan, and Tatyana Rudneva from our team.

The great variety of lymphoid cells was repeatedly emphasized in our previous sections. Among these, two major, functionally different population (i.e., T- and B-lymphocytes) are identified. Which of these two groups should inactivating lymphocytes be assigned to? Or, perhaps, they belong to a third population lymphocytes? In our experiments, irradiated mice were immunized with a mixture of thymocytes (only T-cells) and non-syngeneic bone marrow cells. The colony count performed on the 8th day after transplantation revealed that the inactivation of stem cells was practically complete: not a single colony was formed. One thing was clear: the inactivators are T-lymphocytes. But which of them, mature or immature ones?

Unfractionated, cortisone-sensitive immature thymocytes were replaced by cortisone-resistant mature T-cells—and again the stem cells appeared to be inactivated. An interesting detail: 5,000,000 cortisone-resistant cells induced the same inactivating effect as 50,000,000 unfractionated thymocytes. In other words, the number of cortisone-resistant cells necessary for stem cell destruction is by one order of magnitude more than that of unfractionated thymocytes (Table 5). Since in mice cortisone-resistant cells represent about 10% of the total thymus mass, it may be assumed that inactivators are mature T-lymphocytes.

A comparison of these results with those of Table 3 points to another interesting peculiarity. The inactivating effect of cortisone-resistant thymocytes composed exclusively of T-cells on non-syngeneic stem cells is two times as low as that of unfractionated lymph node cells carrying both T- and B-cells. Let us have another look at Table 3. A practically complete inactivation of non-syngeneic stem cells can be induced by 2,000,000 lymph node lymphocytes, whereas the number of cortisone-resistant cells necessary to yield a response of comparable magnitude should be no less than 5,000,000. Can lymph node B-cells also participate together with T-lymphocytes in the destruction of non-syngeneic haemopoietic precursors?

The role of B-cells in the inactivation of non-syngeneic stem cells was studied in various model systems, using a special strain of mice, the so-called B-mice with artificial T-cell deficiency. To achieve this, the animals are thymectomized and then irradiated with a lethal dose which kills all of their T-cells. As a preventive from the radiation death, the animals receive a transplant of syngeneic bone marrow cells. After this treatment, the lymphoid tissues (spleen, lymph nodes) of such mice do not practically contain T-lymphocytes.

Lymph node B-lymphocytes of B-mice do not display inactivating properties. Administration of these cells to lethally irradiated recipients together with non-syngeneic bone marrow cells left a surviving population of haemopoietic cells that were capable of forming colonies. Similar results were obtained when another methodological approach was used. Lymph node lymphocytes of intact mice were treated *in vitro* with a rabbit antiserum against MTLA, a specific marker for T-lymphocytes, and complement. All of the T-cells in the mixture appeared to be destroyed, but B-lymphocytes survived, unaffected by the treatment. As in previous studies, the surviving B-cells were transplanted to lethally irradiated mice together with non-syngeneic bone marrow cells. And again the stem cells formed numerous colonies: B-lymphocytes failed to deprive them of this ability.

Thus, it has been proven that non-syngeneic haemopoietic precur-

sors are inactivated by T-lymphocytes from various lymphoid organs; a removal of T-cells from a lymphoid cell mixture eliminates the inactivating effect. The inactivation process does not involve B-cells or macrophages (the latter possibility was also examined). T-lymphocytes can themselves aptly destroy rapidly proliferating "non-self" elements; as for B-lymphocytes, they are incompetent as inactivator cells.

**Table 5.** Suppression of Proliferation and Differentiation of Haemopoietic Stem Cells of C57BL Mice by Various Lymphocyte Subpopulations of CBA Mice After Administration of Cell Mixtures to Lethally Irradiated Recipients (CBA × C57BL) $F_1$

| Transplanted cells | | | | CBA/C57BL cell ratio | Percentage of destroyed stem cells |
|---|---|---|---|---|---|
| CBA mice | Number, $\times 10^6$ | C57BL mice | Number, $\times 10^6$ | | |
| Thymus | 5.0 | Bone marrow | 0.2 | 25 : 1 | 99.1 |
| Thymus cortisone-resistant cells | 0.5 | Ditto | 0.2 | 2.5 : 1 | 98.6 |
| | 0.2 | Ditto | 0.2 | 1 : 1 | 46.5 |
| Lymph node lymphocytes | 0.2 | Ditto | 0.2 | 1 : 1 | 98.9 |
| Lymph node B-lymphocytes of B-mice | 5.0 | Ditto | 0.2 | 25 : 1 | 5.8 |
| Lymph node lymphocytes treated with rabbit antisera against: | | | | | |
| MTLA antigen and complement | 0.2 | Bone marrow | 0.2 | 1 : 1 | 0 |
| MBLA antigen and complement | 0.2 | Ditto | 0.2 | 1 : 1 | 33.4 |
| immunoglobulin molecules and complement | 0.2 | Ditto | 0.2 | 1 : 1 | 36.0 |
| Spleen-tropic T-lymphocytes ($T_1$-cells) | 2.0 | Ditto | 0.2 | 10 : 1 | 44.6 |
| Lymph node-tropic T-lymphocytes ($T_2$-cells) | 2.0 | Ditto | 0.2 | 10 : 1 | 63.8 |
| Lymph node lymphocytes of ALS-treated mice | 0.1 | Ditto | 0.2 | 1 : 2 | 0 |
| Lymph node lymphocytes of thymectomized mice | 0.1 | Ditto | 0.2 | 1 : 2 | 70.8 |
| | 0.2 | Ditto | 0.2 | 1 : 1 | 100 |

Despite this categorical statement, the feasible role of B-lymphocytes in the development of the inactivation process still remained obscure. Indeed, B-cells that themselves do not inactivate non-syngeneic haemopoietic stem cells can provide help for T-lymphocytes in this process, i.e., they can function as helpers. Analyzing this possibility, we proceeded from the following assumption. If B-cells are not involved in stem cell inactivation, their removal from the lymph node cell population will have no effect on the inactivating ability of T-lymphocytes. But if they assist T-cells in this process, the inactivating ability of the latter will be diminished after B-lymphocyte removal. In order to subtract B-cells from the mixture, a suspension of lymph node lymphocytes was treated with rabbit antisera against mouse surface antigens specific for B-cells (MBLA) and complement, or against immunoglobulin molecules and complement. Lymph node T-lymphocytes containing no B-cell admixtures in conjunction with non-syngeneic bone marrow cells were administered to irradiated recipients. The results showed a significant diminution of the number of destroyed stem cells. Correspondingly, such treatment dramatically influenced the growth of colonies, which increased by about 60%. The inactivation induced by lymph node T-lymphocytes appeared to be comparable to that evoked by cortisone-resistant thymocytes (Table 5), thus suggesting that the inactivating ability of mature thymus and lymph node T-lymphocytes is not different, as it might be expected, but identical. The differences in the activity of these cells can be interpreted in terms of the accessory function of B-lymphocytes which do not inactivate non-syngeneic haemopoietic precursors but assist T-cells in doing so. This unique, previously unknown property of B-lymphocytes was especially well-pronounced, when other methodological approaches, e.g. electrophoretic separation of T- and B-cells or the use of cell mixtures of different composition, were employed.

But let us better come back to T-lymphocytes. Earlier, we defined thymic lymphocytes capable of inactivating non-syngeneic haemopoietic precursors as mature, cortisone-resistant cells. But how should one characterize the properties of peripheral T-lymphocytes?

Characterization of T-inactivating cells in terms of their tropicity to lymphoid tissues (spleen and lymph nodes) allowed us to relate them either to the $T_1$- or to $T_2$-lymphocyte subpopulations. For this purpose, lethally irradiated CBA recipients were immunized with 40,000,000 unfractionated thymocytes used as a source of T-cells. After a week, the spleens ($T_1$-lymphocytes) and lymph nodes ($T_2$-lymphocytes) of these recipients were removed, and the ability of T-cells to inactivate the haemopoietic stem cells of C57BL mice was tested. The results appeared to be rather unexpected. In lymph nodes, the number of T-inactivators was much greater than in the spleen (Table 5). However, even a 10-fold excess of T-lymphocytes

over non-syngeneic haemopoietic cells would not induce their complete inactivation, although lymph node lymphocytes normally do it at the cell ratio of 1 : 1 (Table 3). Moreover, with a rise in the number of lymphoid cells in the mixture, that of inactivated stem cells did not increase, as it might be expected, but even diminished (Table 6). These data suggest that the inactivating effects

**Table 6.** Comparison of the Ability of Lymph Node Lymphocytes, Spleen- and Lymph Node-Tropic Lymphocytes of CBA Mice to Inactivate Non-Syngeneic Haemopoietic Stem Cells of C57BL Mice

| Ratio of lymphoid cells of CBA mice to bone marrow cells of C57BL mice | Inactivating ability of CBA lymphocytes, % | | |
|---|---|---|---|
| | Lymph node cells of intact mice | Spleen-tropic cells | Lymph node-tropic cells |
| 1 : 5 | 37 | — | — |
| 1 : 2 | 61 | 23.7 | 30.4 |
| 1 : 1 | 82 | 29.6 | 33.3 |
| 2 : 1 | 95 | 35.0 | 49.5 |
| 5 : 1 | — | 47.6 | 74.8 |
| 10 : 1 | — | 44.6 | 63.8 |

of fractionated T-lymphocytes on non-syngeneic stem cells transplanted simultaneously with them are not grossly apparent. What had happened to these cells? Did the fractionation cause their uncoupling or impede their communication and reception of corresponding signals? Or did the fractionation procedure give rise to suppressor cells capable of eliminating the inactivation? Attempting to answer these questions, we conducted the following experiment. Spleen-tropic lymphocytes were mixed in a test-tube with lymphocytes tropic to lymph nodes. To this suspension non-syngeneic bone marrow cells were added, and the resulting mixture was intravenously administered to lethally irradiated mice. Not a single stem cell appeared to be destroyed by the transplanted lymphocytes, so as to judge from the number of colonies formed by these cells. The combination of two lymphoid cell subpopulations not only failed to augment the inactivating effect of these lymphocytes but, on the contrary, eliminated the inactivation altogether. The killing activity of lymphocytes appeared to be blocked by suppressor cells generated by lymphoid organs in the course of fractionation. In this way, the suppressor activity of lymphocytes is directed against their own killer cells, but not against genetically different proliferating stem cells.

G. Ignatyeva who investigated the properties of suppressor lymphocytes came to some interesting results. These cells are absent in athymic animals; besides, they do not carry on their surface the Thy-1 antigen, a specific marker for T-lymphocytes, in particular, for $T_1$-cells. Suppressor lymphocytes do not adhere to glass (which is an attribute of macrophagal cells) and are resistant to ALS. These cells, in contrast with $T_2$-lymphocytes, do not have the ability to recirculate.

But we have again got off the main point, i.e. the properties of T-inactivators. Thymectomy in mice does not seem to affect the functional activity of these cells (Table 5); therefore, they cannot be assigned to the $T_1$-subpopulation. These cells die soon after thymectomy, i.e. they are short-living. Is it $T_2$-lymphocytes, then, that are guilty of "murder"? $T_2$-lymphocytes are mature, long-living cells which carry on their surface a small amount of Thy-1 antigens; they are recirculating cells. Studies in lethally irradiated animals immunized with a mixture of bone marrow cells and lymph node lymphocytes treated with rabbit antiserum against mouse Thy-1 antigen demonstrated that inactivating cells with a low density of Thy-1 antigen cause only partial inactivation of stem cells.

In another experimental series, lymphocytes treated with rabbit antilymphocytic serum (ALS) prior to immunization were unable to inactivate non-syngeneic haemopoietic stem cell precursors (Table 5). These results not only suggest T-inactivators to pertain to the $T_2$-lymphocyte subpopulation, but also confirm the previously made assumption that $T_1$-cells do not possess the killing activity. ALS destroyed all $T_2$-lymphocytes, leaving a surviving population of $T_1$-cells. Should the latter be able to inactivate stem elements, they would kill them all, but in fact, they did not. Therefore, we cannot attribute $T_1$-lymphocytes to the category of inactivating cells. The killing of non-syngeneic haemopoietic precursors is uniquely $T_2$-cell function.

In the sections above, we have told you about our experiments with spleen- and lymph node-tropic T-lymphocytes (i.e., $T_1$- and $T_2$-cells). However, the emergence of suppressor lymphocytes in the course of the fractionation procedure is a considerable obstacle to the analysis. To overcome this difficulty, T-T cell interactions were studied in mixtures of lymph node lymphocytes obtained from thymectomized and ALS-treated mice. In this version of experiment, the inactivating effect of lymphocytes on stem cells was not further amplified, evidencing that the interactions between $T_1$- and $T_2$-cells are not very essential in this case. The crucial role in the inactivation of stem cells belongs to $T_2$-lymphocytes; the assistance of $T_1$-cells is unnecessary. The contribution of B-lymphocytes to the inactivation process, though, is rather significant: under their effects, the number of destroyed stem cells increases more than 2-fold.

$T_2$-Lymphocytes appeared to be staunch soldiers of the Army of Immunity. In some experiments, we irradiated lymphocytes with various doses of penetrating radiation prior to their exposure to non-syngeneic stem cells of lethally irradiated recipients. Naturally, irradiation killed these lymphocytes; as a result, the surviving stem cells formed haemopoietic foci in the recipients' spleens. The higher

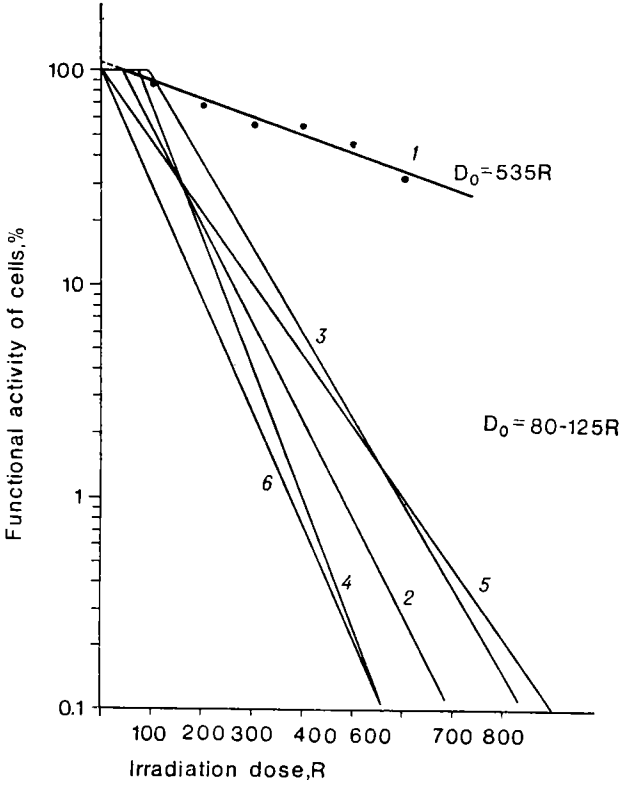



**Fig. 18.** Elimination of functional activity of lymphoid cells by irradiation:

*1*—lymphocyte-induced inactivation of non-syngeneic haemopoietic stem cells; *2*—colony-formation by stem cells; *3*—formation of antibodies against microbial antigens; *4-6*—formation of antibodies against erythrocytic antigens (data from different authors)

the irradiation dose, the greater the number of surviving stem elements and, respectively, the greater the number of colonies formed by them. Notwithstanding, inactivating cells are much more radioresistant than any other rapidly proliferating cells of the immune system (Fig. 18). Different cell functions were characterized in terms of parameter $D_0$, which designates an irradiation dose capable to induce a 37% decrease of the functional activity of these cells.

Whereas for the majority of rapidly proliferating cells, the $D_0$ value varies from 80 to 125 R, that for inactivating lymphocytes is as high as 535 R. Consequently, T-inactivators are more radioresistant than rapidly proliferating cells; therefore, the reproduction processes do not play any conspicuous role in this case. At the same time, the inactivation of non-syngeneic stem cells can be induced only by "living" lymphocytes. After exposure of lymphoid cells to high doses of penetration radiation (10,000 R) their killing ability is eliminated, which also testifies to their relationship to the $T_2$-lymphocyte subpopulation, whose cells are characterized by a relatively high radioresistance.

To sum up all that we know about T-inactivators, we may describe them as mature, radio- and cortisone-resistant, long-living, recirculating, ALS-sensitive cells; these cells do not adhere to glass and survive over 2-3 months after thymectomy. T-inactivating cells carry on their surface a MTLA antigen and a low-density Thy-1 antigen; they are more tropic to lymph nodes than to the spleen. These lymphocytes interact with B-helpers and do not need $T_1$-lymphocytes for the realization of their inactivating effect. They are localized in the thymus, lymph nodes, spleen, and bone marrow, and belong to the $T_2$-lymphocyte subpopulation. The interaction between T-inactivators and non-syngeneic stem cells is not mediated by the GVH response or third population lymphocytes, but is controlled by genes irrelevant to the major histocompatibility complex. When exposed to non-syngeneic stem cells, lymphocytes destroy them, even if the latter differ from them in only one gene. The interactions between lymphocytes and stem cells can be evoked in any part of the circulatory system as well as outside it. Inactivation is a comprehensive process, which can be directed against any non-syngeneic precursor cell engaged in vigorous proliferation and differentiation. In essence, this process is aimed at maintaining the genetic uniqueness of the organism.

### Alteration of the 60 : 30 : 5 Formula

In his report entitled "The Use of a Chromosome Marker for the Study of Stem Cell Inactivation in Mixed Spleen Cell Cultures" presented at the 12th International Congress on Haematology and Blood Transfusion in 1969, the prominent British cytologist, Professor D. Barnes said that in the experiments conducted by R. Petrov and L. Seslavina, spleen cells were obtained from mice of the CBA or C57BL strains. In their studies aimed at the determination of the source of proliferating cells, CBA/H-T6T6 mice were used. The observed inactivation was due to a specific phenomenon of predominant action rather than to the total loss of stem cells... Therefore, their data may be regarded as an additional proof of the

inactivation of colony-forming cells in spleen cell cultures. While the phenomenon discovered by the Soviet immunologists was being scrutinized and verified, the authors of the discovery were already far ahead. On the same day, R. Petrov made a report at one of the Congress sessions, in which he told the participants about a new type of interactions between lymphocytes and haemopoietic stem cells.

All the events described in preceding sections are relevant to the interactions between lymphocytes and genetically different (non-syngeneic) stem cells. But do lymphocytes interact with syngeneic, i.e., genetically identical haemopoietic cells? This question was first answered in the affirmative by a group of Soviet investigators headed by I. Golovistikov. These authors have demonstrated that lymphocytes can also interact with syngeneic stem cells, but instead of killing them they change the direction of their differentiation. Immunization of irradiated hybrid recipients, (CBA × C57BL)$F_1$, with a mixture of bone marrow cells of CBA mice bearing labelled chromosomes (CBAT6T6) and syngeneic lymphocytes whose chromosomes are unlabelled, leads to a diminution of the number of visible colonies formed by the bone marrow cells in the recipients' spleens. This does not happen, however, when a mixture of bone marrow and lymphoid cells of CBA mice is administered to syngeneic irradiated recipients (CBA). But the most striking observation is that in contrast with inactivation, all of the recipients' tissues (lymph nodes, bone marrow, spleen) contain proliferating cells of bone marrow origin. This implies that after transplantation to lethally irradiated recipients of a cell mixture, lymphocytes do not inactivate syngeneic stem cells but change the direction of their differentiation.

According to genetic laws of transplantation, the tissues of (CBA × C57BL)$F_1$ mice are non-syngeneic with regard to the lymphocytes of CBA mice. Within an alien organism, these lymphocytes challenged by large masses of the antigenic material begin "sending orders" to all stem cells compelling them to change their differentiation in the direction of myelopoiesis. The formula of 60 : 30 : 5 thus becomes 0 : 90 : 5. If it is true that stem cells give rise to 60% erythroid, 30% myeloid, and 5% megakaryocyte colonies, none of the stem cell "offsprings" whose differentiation is changed "by the order" of lymphocytes will evolve into erythrocytes; the absolute majority of them will develop into leucocytes. Myeloid colonies are so small in size that they are not even subjected to visual control. Besides, most of them are formed inside the spleen and can be visualized only microscopically, on specially stained histological slides of splenic tissue. By way of example, we may cite one of our experiments conducted in collaboration with V. N. Shvets.

Lethally irradiated CBA mice received a transplant of syngeneic bone marrow cells. The colony count performed on the 8th post-transplantation day revealed 22 splenic colonies (11.7 erythroid

and 6.2 myeloid), i.e., on the average, one myeloid colony was formed for every 1.9 erythroid colonies, the E/M ratio being 1.9. The same ratio was observed, when bone marrow cells of CBA mice were administered to lethally irradiated hybrid recipients (CBA × C57BL)$F_1$. Out of the total colony number of 38, 21 colonies were erythroid; the number of myeloid colonies was half as low (E/M = 2.0). After transplantation of bone marrow cells of CBA mice in conjection with syngeneic CBA lymphocytes (1 : 20) to lethally irradiated CBA recipients, the E/M value was about the same, i.e., 1.8. However, when the same cell mixture was administered to animals of the hybrid strain (CBA × C57BL)$F_1$, the E/M ratio fell drastically—from 2.0 to 0.03! In this case, among the 34.4 colonies, 27.3 were myeloid and only one was erythroid. The remaining colonies were either indistinguishable or of a mixed type. As the number of lymphocytes in the transplant increased 2-fold, i.e., at a 40-fold excess of lymphocytes over bone marrow cells (1 : 40), the erythroid growth was altogether absent, since practically all of the colonies were myeloid.

In this way, under effects of lymphocytes stem cells change their normal course and switch to a predominant formation of myeloid colonies. However, stem cells do not always submit to the will of lymphocytes; they "obey" only those with a highly developed ability to recognize the antigenic material and to be challenged by it. Do you remember our telling you about the fate of stem cells transplanted to non-syngeneic recipients together with lymphocytes? Lymphocytes, which attack and destroy genetically different (non-syngeneic) tissues, including stem cells, behave differently towards the stem cells of their own: they change their differentiation and make them maturate into leucocytes. The latter belong to the category of phagocytic cells whose function is to clear the fragments of killed cells from the battle-field and to ingest foreign particles. Phagocytes lend effective help to lymphocytes in their combat with the foreigners, i.e., stem cells that have challenged their activity.

Lymphocytes are remarkably vigilant against the bone marrow territory. Earlier in this chapter, we repeatedly emphasized the importance of this organ in the context of the whole organism. Suppressor B-lymphocytes safely protect the territory of the bone marrow from immunologic reactions of any kind; presumably, in case of "war", bone marrow cells produce more leucocytes than erythrocytes. Normally, one erythroid colony is formed for every two myeloid colonies (E/M = 0.5). And yet, lymphocytes challenged by non-syngeneic recipient antigens "order" syngeneic haemopoietic stem cells to step off their beaten track and produce more and more leucocytes, for the territory of the bone marrow has to be safely protected from the adversary. As a result, ten instead of

two myeloid colonies for every erythroid colony are formed in the bone marrow cavity: the E/M value decreases, respectively, from 0.5 to 0.1.

In 1970, our hypothesis on the changes in differentiation of bone marrow stem cells under effects of lymphocytes was first confirmed by experiments of Y. Kitamura *et al.* (Japan). These workers reproduced in every detail our experimental pattern and observed a marked shift in the differentiation of stem cells towards myelopoiesis caused by syngeneic lymphocytes.

In late 1970's D. Metcalf *et al.* (Australia) succeeded in isolating soluble factors capable of maintaining prolonged growth of haemopoietic stem cells *in vitro* or changing their differentiation in the direction of myelopoiesis. These soluble factors appeared to be secreted by lymphocytes. In the same years, the first marker antigens controlling the function of haemopoietic precursors were identified on the surface of T-lymphocytes. E. Reinherz and S. Schlossman (USA) discovered an OKT-4 antigen on the surface of human T-lymphocytes; their compatriots, H. Cantor and R. Gershon, were the first to report on Lyt-1, a specific marker of mouse T-cells.

Thus, the results of the Soviet researchers were not only reproduced in many laboratories all over the world, but were further extended in a great number of experimental systems. Some of these will be discussed in subsequent chapters.

## Elimination of Allogeneic Inhibition and Changes in Stem Cell Maturation

Distress signals from a shipwrecked crew leave no one indifferent. At the first sound of SOS signals from a sinking ship, neighbouring vessels put off all other matters, change course, and rush to the rescue—to protect, to aid, to save. So it is with stem cells and lymphocytes. Some genetic combinations of stem cells yield poor growth of colonies in alien organisms. In spleen cells of irradiated recipients, certain allogeneic* donor-recipient combinations of stem cells produce 5 to 10 times fewer colonies than syngeneic ones. In our experiments, immunization of lethally irradiated hybrid mice, (CBA × C57BL)$F_1$, with bone marrow cells of CBA mice had little effect on the ability of stem cells to form colonies; the colony number was only half that in syngeneic (CBA) recipients.

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* A syngeneic transplant is a genetically identical transplant within the same line of genetically identical individuals or enzygotic twins. An allogeneic transplant is a genetically different transplant between individuals of the same species. A xenogeneic transplant is a genetically different transplant between individuals of different species.

Simultaneously, the bone marrow cells of mice belonging to a different parental genotype, C57BL, formed in the irradiated hybrid recipients of (CBA × C57BL)$F_1$ ten times fewer colonies than they did in the syngeneic, C57BL, strain, i.e., 4.0 and 40.2 colonies, respectively. In 1964, the Swedish investigators K. Hellström and G. Möller introduced the term "allogeneic inhibition" to describe the repression of growth of a transplant (e.g., skin, tumour, haemopoietic cells, etc.) of a parental genotype within a hybrid recipient.

An interesting finding was reported in 1970 by J. Goodman and G. Grubbs (USA) who studied allogeneic inhibition of haemopoietic stem cells. It appeared that immunization of irradiated hybrid recipients ($F_1$) with bone marrow cells in combination with syngeneic thymus T-lymphocytes caused a drastic rise in the number of colonies formed by the stem cells and eliminated their allogeneic inhibition. In this way, T-lymphocytes "save" syngeneic stem cells from the inevitable death by helping them to survive and make colonies. Receiving signals of distress from stem cells, lymphocytes consummate their function.

The elimination of allogeneic inhibition can be easily reproduced with lymphocytes from different strains. In 1971, we conducted a series of experiments with hybrid recipients of different genotypes—(CBA ×C57BL)$F_1$, (C57BL × DBA/2)$F_1$, and (A × C57BL)$F_1$. In all cases, lymph node lymphocytes of C57BL mice restored the interrupted growth of colonies induced by syngeneic bone marrow cells of the same genotype. Is the process of stem cell maturation the same as that in the cells that are not subjected to allogeneic inhibition? Do antigen-stimulated lymphocytes always dictate their will to stem cells, or are lymphocyte-induced changes in stem cell differentiation specific only for some, perhaps, unique, donor-recipient combinations?

Attempting to answer these questions, we conducted a number of experiments that have already been discussed in previous sections. Bone marrow cells of the parental genotype (C57BL) were administered to lethally irradiated hybrid recipients (CBA × C57BL)$F_1$. The number of colonies formed in the spleens of the irradiated animals was only four, i.e., ten times as few as that in the irradiated syngeneic mice of C57BL. However, the direction of differentiation of surviving stem cells was unaffected by allogeneic inhibition: one myeloid colony was formed for every three erythroid colonies under these conditions. An addition of syngeneic lymphocytes to the bone marrow transplant changed dramatically the further fate of the stem cells. The number of myeloid colonies rose drastically; that of erythroid colonies also increased, although insignificantly. As a result, the direction of differentiation of haemopoietic precursors changed from predominantly erythroid to predominantly myeloid, and the total number of colonies increased. Similar results

were obtained in experiments with $(A \times C57BL)F_1$ mice.

Thus, as early as 1972, it became evident that elimination of allogeneic inhibition of stem cells by lymphocytes is associated with a switch-over to an increased production of myeloid colonies. Lymphocytes protect stem cells and save them from death. But the neighbourhood of large masses of antigenic material requires quantities and quantities of leucocytes. At this point, lymphocytes bend stem cells to their will and make them produce myeloid colonies.

Which of lymphocyte subsets are responsible for these changes? Do the same or different lymphocytes stimulate the growth of stem elements, protect them from death and "order" them to differentiate in the direction of myelopoiesis? Khachik Sayadyan (USSR) attempted to approach this problem by comparing the ability of lymphocytes to stimulate the proliferation of haemopoietic precursors and eliminate their allogeneic inhibition, on the one hand, and to change the differentiation of stem cells, on the other. These experiments revealed substantial differences between lymphocytes: the former appeared to be resistant to irradiation and inhibitors of protein and nucleic acid synthesis, while the latter were not.

In 1974, our results were confirmed by experiments of N. Basford and J. Goodman (USA), who demonstrated that syngeneic lymphocytes are capable of changing the differentiation of stem cells. Lethally irradiated mice, $(C57BL/6 \times DBA/2)F_1$ and $(C57BL \times C3HAnf)F_1$, were immunized with a mixture of bone marrow cells of C57BL mice and syngeneic thymus or lymph node lymphocytes. Apart from stimulating the colony growth, the syngeneic lymphocytes changed the direction of stem cell maturation, i.e. effected the transition from the predominantly erythroid to the predominantly myeloid growth. These authors succeeded in discriminating between lymphocytes from different tissues (thymus, lymph nodes) and drew lines between lymphocytes that stimulate the growth of stem cells and those that change the differentiation of haemopoietic precursors. Lymph node lymphocytes irradiated *in vivo* with a dose of 900 R lost their ability to change the differentiation of haemopoietic precursors, but influenced the colony production in much the same way as did non-irradiated cells. In contrast with peripheral lymphocytes, irradiated thymus cells had no profound influence on the colony growth.

Thus, line has been drawn between lymphocytes that control the differentiation of haemopoietic stem cells and those eliminating their allogeneic inhibition. These lymphoid cells pertain, in all probability, to different lymphocyte subpopulations. In 1978, J. Goodman *et al.* (USA) suggested assigning the lymphocytes with the ability to stimulate the growth of stem cells and to eliminate their allogeneic inhibition, to the category of amplifiers. The growth-stimulating cells were attributed to the $T_2$-subpopulation.

## Inactivation or Changes in Differentiation?

Professor A. Poverenny (USSR) focussed his attention on experiments with rabbit antisera, whose use was reported by the immunologist from the USA E. Golub. Such antisera obtained by immunization of rabbits with mouse brain antigens appeared to be highly specific not only against the immunizing antigens, but against mouse T-lymphocytes as well. Mouse brain tissue contains an antigen closely resembling that of Thy-1, a specific marker for T-lymphocytes; therefore, antibrain antisera can specifically interact with mouse T-cells. The source of tissue (i.e., nervous, lymphoid, etc.) is unimportant for the antisera; for them, the main thing is the antigen capable of binding their specific antibodies. E. Golub showed that antisera against mouse brain antigens can interact with nervous cells of a great variety of animal species, e.g., rats, guinea pigs, cats, rabbits, goats, pigs, sheep, dogs, and horses. But the most important finding was that after incubation with the antisera, the bone marrow cells of mice lost their ability to proliferate and form colonies in the spleens of lethally irradiated recipients. At the same time, after pre-incubation with thymic lymphocytes, the antisera retained their ability to eliminate the colony-forming activity of bone marrow stem cells. These data have major implications which can be formulated as follows. Mouse antibrain antisera contain antibodies that are specific against haemopoietic stem cell antigens. Thymic cells do not seem to carry such surface antigens, since their pre-incubation with the antisera do not influence the activity of the latter.

These results were first published in 1972 and gave an impetus to a long series of publications concerned with the study of surface macromolecules of haemopoietic stem cells. These works led to another important discovery. But before we come to the consideration of these data, we would like to make some preliminary remarks. At present, *in vitro* cultivation of haemopoietic cell colonies has become a commonplace, technically uncomplicated procedure. By adding bone marrow cells to a culture medium containing erythropoietin, stimulating the maturation of haemopoietic precursors into red blood cells, one can induce intensive growth of erythroid colonies. In the presence of the colony growth stimulating factor *in vitro* (see the chapter "Molecular Messengers of Immunity"), haemopoietic precursors differentiate into cells of a myeloid type which, in turn, give rise to granulocyte colonies. Using appropriate techniques, one can induce the growth of megakaryocyte colonies, i.e. those further evolving into thrombocytes, or lymphoid cell colonies predominantly containing T- or B-lymphocytes.

It appeared that mouse antibrain antisera do not affect the *in vitro* growth of myeloid colonies. Treated or untreated, bone marrow

haemopoietic stem cell precursors are always in perfect condition and ready to proliferate and form granulocyte colonies. However, as we have seen in preceding sections, administration of such antisera to mice eliminates the colony-forming activity of bone marrow cells: the number of colonies formed in the recipients' spleens falls drastically. These results have led us to the only plausible conclusion that the precursor cells giving rise to myeloid colonies *in vitro* are antigenically different from those forming colonies in the spleens of lethally irradiated recipients, i.e. from haemopoietic stem cells.

In a concise form the above-described results may be presented as follows. Antisera directed against brain antigens specifically inactivate certain types of precursor cells, leaving others unaffected. This viewpoint had been widely spread among immunologists until mid-1970's, when A. Poverenny *et al.* made the first breach in the "stronghold of conceptions". These authors contemplated a very simple problem: if certain antigens are shared between the brain and T-lymphocytes, and the antisera directed against brain antigens eliminate the proliferation of stem cells, the latter may not be inactivated by these antisera. Can antibrain antisera inactivate T-helpers, i.e., the cells that assist stem cells to form colonies? In the experiment, two groups of lethally irradiated recipients were used, one which was immunized with bone marrow cells pre-treated with mouse antibrain antiserum, and one which received a transplant of antiserum-treated bone marrow cells in conjunction with syngeneic thymus T-lymphocytes. Expectedly, in the first group the colony growth was absent, while in the second one lymphocytes eliminated the inactivating effect of the antiserum and restored the colony growth. A similar, although far less pronounced, effect was evoked by thymosin, a humoral factor of the thymus. Simultaneously but independently of A. Poverenny, the British investigators N. Testa and R. Schofield obtained very similar results. In their experiments, the restoration of colony growth was due to the addition of T-lymphocytes to bone marrow cells pre-treated with an antibrain antiserum. It appears, therefore, that the antiserum does not inactivate stem cells proper but is directed against lymphocytes involved in the colony production. An addition of thymus cells eliminates the deficiency of stem cells. The interaction between T-helpers and stem cells seems to play a crucial role in the colony formation process.

This discovery resulted in an outburst of new ideas. In the experiments by E. Golub, incubation of antibrain antisera with thymus cells did not eliminate their activity. Maybe thymocytes contain too few helper T-cells? If it is true, these cells will be unable to bind all of the antibodies present in the antiserum. Perhaps, the incubation has no effect on the antiserum but influences the activity of thymocytes? A. Poverenny examined this possibility and found

that treatment of thymus cells with antibrain antisera deprives the former of their ability to restore the interrupted growth of colonies. This finding strongly suggests that the percentage of helper cells in the thymocyte population is very low indeed. These data were confirmed by studies from other laboratories. A. Poverenny and D. Kaulen (USSR) simultaneously demonstrated that treatment of bone marrow cells with mitogens (vegetative substances capable of selectively stimulating the proliferation of T-cells) eliminates their colony-forming ability. For mitogens, D. Kaulen used phytohaemagglutinin; A. Poverenny treated bone marrow cells with concanavalin A. In both cases, the colony growth was impaired and could be restored by an addition of thymus cells to mitogen-treated bone marrow cells. This effect, however, was observed only when thymus cells were in excess: 10,000,000 thymocytes added to concanavalin A-treated bone marrow cells restored the normal growth of colonies, while 100,000 thymocytes added to bone marrow cells treated with phytohaemagglutinin did not. Presumably, the percentage of T-lymphocytes with a high helper activity with respect to haemopoietic precursors is rather low. Besides, mitogen-challenged cells do not seem to "notice" their distressed "kinsmen": they have to take care of themselves and keep their own proliferation going.

When contemplating these results, we tried to put the afore-mentioned events in a somewhat different light under the stipulation that antisera eliminate the growth of colonies (E. Golub) and that thymus cells can also play a role in the restoration of this process (A. Poverenny, N. Testa and R. Schofield). Should these data be interpreted in terms of T-lymphocyte control over stem cell differentiation but not over their proliferation? If that were true, the events described above would take a different course. T-lymphocytes present among bone marrow cells will change the differentiation of stem elements in the direction of predominant formation of erythroid colonies. Treatment of bone marrow cells with mouse antibrain antisera will partly eliminate T-cells, namely those "ordering" stem cells to differentiate into erythroid colonies. The whole process of stem cell maturation will be thus reversed and switched from the predominantly erythroid to the predominantly myeloid growth. As a rule, myeloid colonies are inaccessible to visual control; therefore, the number of visible colonies will be diminished. After addition of T-lymphocytes, the normal differentiation of stem cells in the direction of erythropoiesis will be restored, resulting in the appearance of visible colonies in the recipients' spleens. All this is strongly suggestive of the existence of two types of T-lymphocytes that effect their control over the maturation of stem cells, i.e., those which change the direction of differentiation of haemopoietic precursors towards erythropoiesis and those which direct it towards myelopoiesis. The former are highly sensitive to the effects of antibrain

antisera. It is thus conceivable why the haemopoietic precursors capable of forming myeloid colonies *in vitro* are resistant to the antisera.

In order to test this possibility, Akram Batyrbekov (USSR) immunized lethally irradiated hybrid mice, (CBA × C57BL)$F_1$, with syngeneic bone marrow cells, both treated and untreated with a rabbit antiserum against mouse brain antigens *in vitro*. In this instance, the number of visible colonies formed by the antiserum-treated cells in the recipients' spleens would be expected to decrease more than 3 times, i.e., from 10.3 to 2.8. However, the microscopic analysis revealed a greater number of colonies, largely at the expense of myeloid colonies that cannot be detected visually, but the direction of the differentiation process did not change. A differential colony count revealed that the number of erythroid colonies in the antiserum-treated cells decreased markedly—from 8.8 to 1.9, i.e. 4.6-fold, while that of myeloid colonies showed only a slight decrease—from 9.0 to 7.2, i.e. 1.2-fold. The value of the E/M ratio decreased, respectively, from 1.0 to 0.26. After addition of syngeneic thymocytes to the antigen-treated bone marrow cells, the haemopoiesis was restored in both cases. The normalization of the colony growth was largely due to the predominant formation of erythroid colonies; the myeloid growth was also increased, although insignificantly. Presumably, thymus cells contain T-lymphocytes of two types—those which change the direction of maturation of stem cells towards erythropoiesis and those which make them maturate via myelopoiesis. An addition of thymocytes led to an increase in the number of both erythroid and myeloid colonies. However, despite the marked tendency for the increased colony growth, the E/M ratio was restored only partly, and its value did not exceed 0.48. Obviously, the hypothesis on the existence of two lymphocyte subsets changing the differentiation of stem elements in the direction of erythropoiesis (production of erythrocytes) or myelopoiesis (production of leucocytes) needs clarification and experimental verification. Notwithstanding, the use of antibrain antisera provides, beyond dispute, entirely new outlooks in the study of intimate mechanisms underlying the interactions between lymphocytes and haemopoietic stem cells as well as in elucidation of regulatory influences of T-lymphocytes on the haemopoietic system.

By analogy, we may cite another trend of research that seems to be of great importance. In 1978, S. Sharkis *et al.* (USA) demonstrated that certain T-cell deficiencies are associated with changes in normal differentiation of haemopoietic cells, in particular, those developing into red blood cells (erythrocytes). The experiments were carried out in $w/w^v$ mice with macrocytic anemia, an inborn genetic deficiency concomitant with lack of erythrocytes. This haemopoietic disorder was previously associated with the inability of stem cells to devel-

op into red blood cells. But could macrocytic anemia be the result of T-cell deficiency rather than stem cell inability? Bone marrow cells of healthy donors were transplanted to nonirradiated recipients ($w/w^v$). The stem cells of the transplant developed on a satisfactory basis within the recipients' organisms and produced multiple colonies, predominantly of an erythroid type (E/M = 1.6). Treatment of intact bone marrow cells with an antiserum against Thy-1 antigen specifically directed against T-lymphocytes did not influence the total growth of colonies, but changed the direction of differentiation of haemopoietic precursors towards myelopoiesis. Correspondingly, the E/M ratio changed from 1.6 to 0.17. An addition of syngeneic thymocytes obtained from healthy donors to the antiserum-treated bone marrow cells restored the haemopoiesis; the E/M ratio returned to the normal value—from 0.17 to 1.8. The thymocytes of $w/w^v$ mice did not induce such an effect. The cells having the ability to change the differentiation of haemopoietic precursors in the direction of erythropoiesis were called by S. Sharkis "antitheta-sensitive regulatory cells", or TSRC. There is an opinion that the interaction between TSRC and stem cells is a necessary step in the overall process of erythropoiesis in healthy individuals. Thus, T-lymphocytes (TSRC) provide a way to normalize the proliferation and differentiation of haemopoietic stem cells and repair haemopoiesis in $w/w^v$ mice.

The results of experiments with treatment of bone marrow cells with mouse antibrain antisera and antisera against antigen Thy-1 showed a good coincidence. In both cases, the expulsion of T-lymphocytes from bone marrow cell suspensions resulted in impaired erythropoiesis. The normal differentiation of stem elements was restored by an addition of a T-lymphocyte excess. It follows, therefore, that the predominant growth of myeloid colonies in the recipients' spleens can be achieved in two ways, i.e., either by additions of antigen-challenged peripheral lymphocytes to intact bone marrow cells or by expulsion of T-lymphocytes from bone marrow cell suspensions. In all probability, there exist two different lymphocyte subpopulations that direct the differentiation of stem cells either towards erythropoiesis or towards myelopoiesis.

The exact number of lymphoid cell subpopulations responsible for the interaction with syngeneic stem cell precursors remains to be established. This problem requires detailed analyses of various lymphoid cells involved in the control over haemopoiesis. The properties of lymphocytes changing the differentiation of haemopoietic precursors in the direction of erythropoiesis are currently obscure. According to our data (Kh. Sayadyan), T-lymphocytes that normalize the functions of antibrain antiserum-treated bone marrow cells are sensitive to irradiation (600-800 R) and resistant to some inhibitors of protein and nucleic acid synthesis (e.g., cyclophospha-

mide, actinomycin D, mitomycin C). A. Poverenny holds the view that these cells should be assigned to the category of T-lymphocyte precursors.

When studying the properties of TSRC, S. Sharkis showed that the percentage of these cells in the bone marrow is very small ($\leqslant 1\%$); these lymphocytes are resistant to high irradiation doses (1050 R); their removal deprives bone marrow cells of their prime property, i.e., self-reproduction. Therefore, T-lymphocytes are not only necessary for the normal maturation of stem cells, but also for the realization of their main function. This characteristic feature of precursor cells, as we have seen, is inherent in them as in stem elements.

The properties of lymphocytes changing the direction of stem cell differentiation towards myelopoiesis have been studied in more detail.

### T-Differentiating Lymphocytes

While seeking for a good illustrative way of presenting the factual material that would provide a truly satisfying account—from the point of view of thoroughness—of the properties of lymphoid cells interacting with syngeneic haemopoietic precursors and directing their differentiation towards myelopoiesis, we made up a summary table (Table 7).

This table, being amply supplied with descriptive definitions, bears only a remote resemblance to reference tables used by chemists or mathematicians. At the same time, such a way of presenting one's experimental data makes it possible to omit insignificant details. The more so that the techniques routinely used for fractionation of lymphocytes and the properties of lymphoid subpopulations so produced have already been described in preceding sections. In Table 7, we only provide a sketch of lymphoid cell populations with reference to their doses and E/M values which reflect the direction and magnitude of changes in stem cell differentiation.

Normally, upon transplantation of bone marrow cells without lymphocytes, the value of the E/M ratio is 2-3. After addition of lymphocytes to the bone marrow cell transplant, this value drops below 1, which points to the changes in the direction of differentiation of haemopoietic precursors from the predominantly erythroid to the predominantly myeloid growth.

In our experiments, we used CBA mice as bone marrow and lymphocyte donors, and lethally irradiated hybrid mice, (CBA $\times$ $\times$ C57BL)$F_1$, as recipients. The colony count was performed on the 8-9th post-transplantation day. In a special set of experiments, the colony estimates made on the 7-8th and 10-12th days after the transplantation of the cell mixture were compared. The results appeared

**Table 7.** Effects of Various Lymphocyte Subpopulations on the Direction of Differentiation of Syngeneic Bone Marrow Haemopoietic Stem Cells

| Transplanted lymphocytes of CBA mice and their number, $\times 10^6$ | Ratio of bone marrow cells to lymphocytes in cell mixtures | E/M ratio |
|---|---|---|
| — | — | 2.4 |
| From lymph nodes, 2.0 | 1 : 20 | 0.17 |
| B-lymphocytes from lymph nodes of B-mice, | | |
| 2.0 | 1 : 20 | 4.75 |
| 4.0 | 1 : 40 | 2.38 |
| From thymus, | | |
| 2.0 | 1 : 20 | 1.71 |
| 20.0 | 1 : 200 | 0.5 |
| Cortisone-resistant thymus cells, | | |
| 2.0 | 1 : 20 | 1.33 |
| 4.0 | 1 : 40 | 0.47 |
| 20.0 | 1 : 200 | 0.10 |
| Cortisone-resistant lymph node cells, 2.0 | 1 : 20 | 0.25 |
| From lymph nodes of thymectomized pubescent mice, | | |
| 2.0 | 1 : 20 | 0.19 |
| 4.0 | 1 : 40 | 0.07 |
| Spleen T-cells of T-mice, 2.0 | 1 : 20 | 1.42 |
| H-2 antigen-stimulated spleen T-cells of T-mice, 2.0 | 1 : 20 | 1.0 |
| H-2 antigen-stimulated lymph node cells of T-mice, 2.0 | 1 : 20 | 0.5 |
| From lymph nodes of ALS-treated mice | 1 : 20 | 2.4 |

*Note*: In all experiments, 100,000 bone marrow cells of CBA mice were administered to lethally irradiated recipients (CBA×C57BL)$F_1$, simultaneously with lymphocytes.

to be essentially the same, and the experimental pattern reproduced, with the exception of the mouse strains, that of the foregoing experiments (Fig. 12).

Who is who, then? Upon addition of lymph node lymphocytes to syngeneic bone marrow cells, the E/M ratio decreases from 2.4 to 0.17. But lymph node lymphocytes contain both T- and B-cells. Which of them give orders to stem cells? We conducted some experiments with B-mice whose lymphoid tissues are devoid of T-lymphocytes. As can be seen from Table 7, 2,000,000-4,000,000 lymph node B-lymphocytes do not change the differentiation of stem cells. Can T-lymphocytes do it? A yes answer seems to be the most plausible one, but it demands further experiment. The use of thymus lymphocytes is the simplest way. We have seen, in preceding chapters,

that the thymus is a lymphoid organ which does not contain B-lymphocytes, but literally swarms with T-cells. Thymus lymphocytes are, indeed, endowed with "commander" features, but these are rather unostentatious. 2,000,000 thymocytes appeared to have practically no effect on the differentiation of stem cells. Changes in the direction of this process could be induced only when the number of thymus cells in the transplant was 10 times as great, i.e., 20,000,000. Only then did the stem cells step off the path of erythropoiesis and switched to a large-scale production of myeloid colonies. Is it the result of different activities of T-lymphocytes obtained from various sources (lymph nodes, thymus) or do the transplanted thymocytes contain too few mature T-cells?

Before we come to answer this question, let us interrupt our discussion for a moment. The problem raised hereby is relevant to an entirely new level, i.e., a study of lymphocyte subpopulations. Engrossed with our matter, we did not notice our having passed from the population to the subpopulation level. What conclusions can be drawn now? For one thing, B-lymphocytes do not change the direction of differentiation of haemopoietic stem cells. The dictator's function is the privilege of T-lymphocytes which outline the pathways of differentiation of haemopoietic precursors and thus control haemopoiesis. From preceding sections, you know already about the events that take place after administration of lymphocytes together with non-syngeneic stem cells to lethally irradiated recipients: lymphocytes destroy stem elements unassisted by B-cells. But the latter are in no way "indifferent" to what happens, and they provide help for T-lymphocytes in the destruction of foreign target cells. What is the role of B-cells in the T-lymphocyte interactions with syngeneic stem cells? Do they help T-lymphocytes under these conditions? So far, there has been no unequivocal answer to this question. Our preliminary data, however, do not exclude this possibility. But let us better go on with our story.

Cortisone-resistant mature thymus cells added to bone marrow stem cells instead of unfractionated thymocytes also change the direction of stem cell maturation; their activity, however, is five times as great as that of unfractionated cells. Transplantation of 20,000,000 thymus cells and 4,000,000 cortisone-resistant thymocytes yields a E/M ratio of 0.47-0.5. But only when the number of mature T-cells in the transplant increases up to 20,000,000, the E/M value becomes comparable to that observed after administration of 2,000,000 lymph node cells to lethally irradiated recipients. This implies that the activity of lymph node lymphocytes is ten times as high as that of mature thymus cells. Do B-lymphocytes provide help for peripheral T-cells in this interaction and thus sustain their dictator's function? Once such suspicion has been aroused, the "case" will be investigated.

What else do we know about T-lymphocytes which control the direction of stem cell maturation? An essential feature of T-cells is their sensitivity to thymectomy, i.e. thymus removal in pubescent animals. This surgery is associated with rapid elimination of short-living lymphocytes ($T_1$-cells); long-living lymphocytes assigned to the $T_2$-subpopulation gradually die off at later times (Table 1). Thymectomy has no effect on the differentiation process: lymph node lymphocytes of thymectomized donors cause the same effect on stem cell maturation as those of intact donors. Therefore, lymphocytes possessing the ability to change the maturation of haemopoietic precursors can be related to $T_2$-cells. They are mature, cortisone-resistant, long-living, recirculating cells that are tropic to lymph nodes (but not to the spleen).

In a special set of experiments using syngeneic and non-syngeneic recipients, T-lymphocytes were characterized in terms of their tropicity to the spleen ($T_1$-cells) and lymph nodes ($T_2$-cells). T-lymphocytes of CBA mice which migrated to the spleens of syngeneic (CBA) or non-syngeneic ($F_1$) recipients had no effect on the maturation of stem cells (E/M = 1.0-1.42). At the same time, lymph node lymphocytes of the hybrid recipients changed the differentiation of precursor cells in the direction of myelopoiesis (E/M = 0.5). These data confirm the hypothesis suggesting that maturation of haemopoietic precursors in the direction of myelopoiesis is controlled by $T_2$-lymphocytes.

Earlier in this chapter, we told you that the interaction between T-inactivating lymphocytes and non-syngeneic stem cells is not mediated by the recipient's organism. But how do T-lymphocytes treat syngeneic stem cells: in the same way as T-inactivators do, or differently, via the GVH response? Let us have another look at Table 7. T-lymphocytes that are tropic to lymph nodes and challenged by hybrid recipients' antigens, "order" syngeneic stem cells to change the direction of their maturation towards myelopoiesis. The activity of lymph node-tropic lymphocytes, however, is much lower than that of unfractionated lymph node cells (E/M = 0.5 and 0.17, respectively). Therefore, the observed shift in the differentiation of precursor cells is not mediated by the response of the non-syngeneic tissues of the recipient to the lymphocyte "attack", otherwise, the changes in the direction of myelopoiesis would be incompatible with the activity of antigen-unchallenged lymphocytes. No such effect was observed when antigen-stimulated lymphoid cells tropic to the spleens of lethally irradiated hybrid recipients were used.

In the sections above, we have told you about our search for lymphocyte subpopulations responsible for the inactivation of non-syngeneic stem cells and delimited the duration of this process by a period of 4-5 days. This time can be reduced to 24 hours, when antigen-challenged, spleen-tropic lymphocytes are used for immu-

nization of lethally irradiated recipients of hybrid strains. Such lymphocytes develop an extraordinarily strong activity against non-syngeneic tissues of the recipient. Yet, antigen-challenged lymphocytes differ only insignificantly from antigen-unchallenged spleen tropic ones and are even less active than unfractionated lymph node-tropic T-cells. It may be thus concluded that the interaction between $T_2$-lymphocytes and syngeneic haemopoietic stem cells is not mediated by the GVH response. The observed changes in the differentiation of haemopoietic precursors towards myelopoiesis are rather due to the direct interaction of stem cells with lymphocytes challenged by tissue antigens. This observation can be illustrated by the following data. Immunization of lethally irradiated recipients with 2,000,000 cortisone-resistant thymocytes of the parental strain yields a strong GVH response. The transplanted lymphocytes attack the non-syngeneic tissues of the recipients and bring them to destruction, terminating in the death of animals. However, the given dose of cortisone-resistant thymocytes is too low to change the direction of stem cell differentiation towards myelopoiesis (Table 7); this process requires larger amounts of lymphocytes. Accordingly, the GVH response should be omitted as a possible cause of the observed changes. This effect is, in fact, mediated by T-lymphocytes which directly interact with haemopoietic precursors.

In 1978, R. Petrov and V. Man'ko characterized the lymphoid cells controlling the differentiation of haemopoietic stem cells as T-differentiating lymphocytes ($T_D$). Earlier, lymphocytes with similar functions were termed by S. Sharkis *et al.* as antitheta-resistant regulatory cells (TSRC). In the same year of 1978, J. Goodman *et al.* suggested the term "T-amplifiers" to describe T-lymphocytes stimulating the proliferation of stem elements. One characteristic feature of $T_D$-lymphocytes is their high sensitivity to irradiation and inhibitors of protein (cycloheximide) and DNA (mitomycin C) synthesis. Irradiation of lymphoid cells with 200 R or their treatment with the above-mentioned metabolites eliminates their ability to change the differentiation of syngeneic stem cells in the direction of myelopoiesis. Similar results were obtained, when ALS was administered to lymphocyte donors or lethally irradiated recipients immediately after the transplantation of a cell mixture (Table 7). $T_D$-lymphocytes appear to be much more sensitive to irradiation than T-inactivators: the $D_0$ values for these lymphocytes are 110 and 535 R, respectively (Fig. 19). These data aptly demonstrate that the process of differentiation of haemopoietic stem cells is controlled by living radiosensitive T-lymphocytes. Cell division and protein synthesis also play a role in the manifestation of the $T_D$-lymphocyte activity.

In many details, the interaction between $T_D$-lymphocytes and syngeneic haemopoietic precursors reminds one of the events that

take place during T-cell interactions with genetically different (non-syngeneic) stem cells. Both types of interactions are relevant to the ability of stem cells to proliferate and form colonies. In both cases, T-lymphocytes tend to impose their "will" on stem elements: they submit them to their authority and suppress their vitality. T-lymphocytes terminate the life-cycle of non-syngeneic stem cells

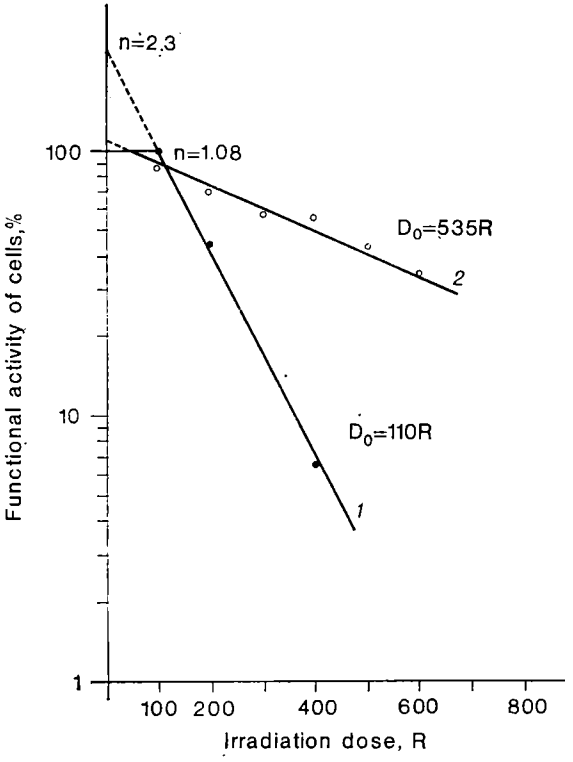



**Fig. 19.** Comparison of sensitivity of T-differentiating (*1*) and T-inactivating (*2*) lymphocytes to irradiation.

and change the differentiation of those stem elements that are syngeneic to them. Both processes occur simultaneously, and their outcome depends heavily on the activity of T-inactivating or T-differentiating lymphocytes. Therefore, the ultimate utility of our experimental system is not confined to the characterization of $T_D$-lymphocytes; this system can be also a helpful guide in a search for effective means of selective suppression or augmentation of regulatory activity of lymphoid cells. The elimination of effects of $T_D$ by irradiation and administration of ALS or inhibitors of protein and DNA synthesis provides compelling evidence for this assumption.

Thus, $T_D$-lymphocytes can be characterized as mature, cortisone-resistant, long-living, radio- and ALS-sensitive, thymectomy-independent, recirculating, lymph node-tropic cells that are highly sensitive to inhibitors of protein and DNA synthesis; their interactions with stem cells are not mediated by the GVH response. These lymphocytes need antigenic stimulation for their activity; the degree of this stimulation, however, does not influence their ability to change the differentiation of stem cells in the direction of myelopoiesis. In many features, the "dossier" of $T_D$-lymphocytes resembles that of T-inactivating cells: both lymphocytes belong to the $T_2$-subpopulation and are mature, long-living, cortisone-resistant, lymph node-tropic cells that are sensitive to ALS and insensitive to thymectomy. Their effects on stem cells are not mediated by the GVH response; both of them influence stem cells directly. At the same time, these two lymphocyte subpopulations differ essentially in a number of features. $T_D$-lymphocytes are localized in the radiosensitive, while T-inactivators—in the radioresistant fraction of $T_2$-cells. Besides, antigen-challenged T-inactivating lymphocytes exhibit an exceedingly strong activity towards non-syngeneic stem cells and eventually bring them to destruction. $T_D$-lymphocytes do not possess such capabilities. Their activity is either unaffected by excessive antigenic stimulation or is even diminished. There are also some minor differences between these two lymphocyte subpopulations.

## T-Cell Deficiencies and Their Consequences

Nataliya Aleynikova (USSR) devoted much attention to the study of various immunodeficiencies, i.e. pathological conditions associated with impairments of certain links in the immune system, with the emphasis primarily on the after-effects of these disturbances of the T-system of immunity. If it is true that lymphocytes "order" stem cells to differentiate in the direction of myelopoiesis (predominant production of leucocytes), how will the organism respond to the shortage of lymphocytes? As we know already, lymphocytes are produced, for the most part, in the thymus, the central organ of the immune system. A surgical removal of this organ leads to severe disorders, in the first place, to T-immunodeficiencies. In the experiments carried out by N. Aleynikova in 1976, the animals underwent thymectomy. A week passed, then another week, a third, a fourth ... The thymectomized animals gradually developed a T-lymphocyte deficiency. How is thymectomy reflected on the bone marrow cells of such animals? In order to clarify this problem, N. Aleynikova used the approach that has been described at the beginning of this chapter, i.e., partial protection of the bone marrow of experimental animals upon irradiation by lead plate screening. Stem cells which migrate from the bone marrow and circulate with

the blood are diffused throughout the organism, and they form haemopoietic foci in the tissues where their number is low, i.e., in the spleen and the damaged parts of the bone marrow.

It has been shown that in T-deficient mice, the rate of stem cell migration from the protected parts of the bone marrow is sharply

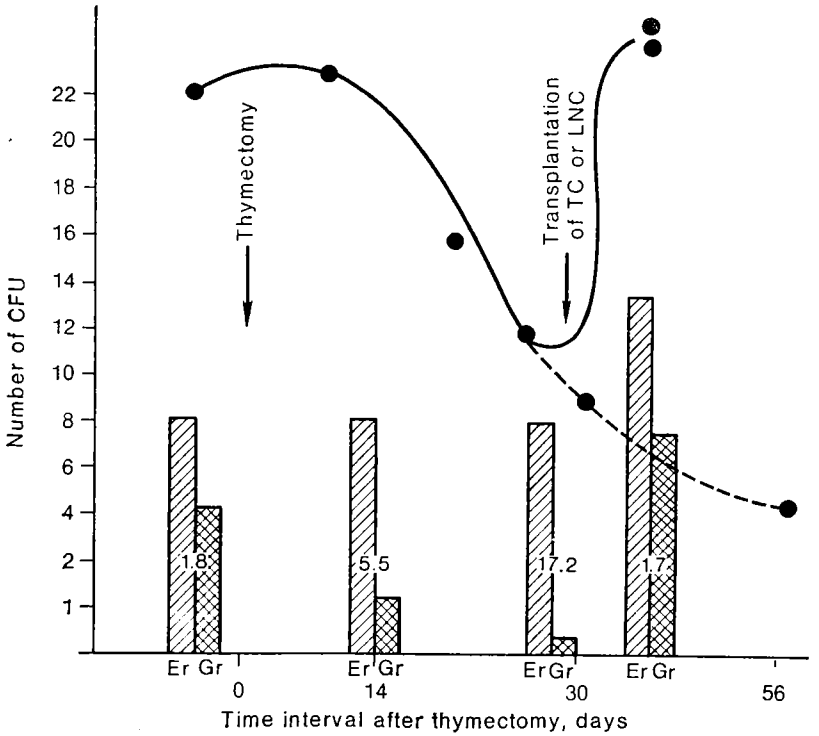



**Fig. 20.** Migration and differentiation of haemopoietic stem cells of T-deficient (thymectomized) mice and restoration of T-deficiency by administration of thymus and lymph node lymphocytes.

CFU—colony-forming units; TC—thymus cells; LNC—lymph node cells.
The columns designate the number of erythrocyte (Er) and granulocyte (Gr) colonies and their ratios; the curved line designates the number of colony-forming cells migrating from the screened part of the bone marrow of lethally irradiated recipients to the spleen

decreased. Eight weeks after thymectomy, the number of splenic colonies is six times as low as that of control (Fig. 20). Besides, the migrating stem cells of T-deficient mice lose their ability to form myeloid colonies. The 60 : 30 : 5 ratio changes into 90 : 5 : 3. Erythropoiesis becomes the predominant form of haemopoiesis, resulting in an excessive production of red blood cells. Within one month after thymectomy, the E/M ratio rises from 1.8 to 17.2! Indeed, the interference of lymphocytes seems to be vitally important

for myelopoiesis. T-Lymphocyte deficiency can be easily removed by intravenous injections of thymus or lymph node cells which augment the recirculation of stem cells from the bone marrow into the blood and thus restore the myeloid haemopoiesis. As a result, the E/M ratio drops from 17.2 to the normal value of 1.7, and the formula of 60 : 30 : 5 becomes predominant. The same effect is observed when thymosin, a thymus hormone, is intravenously injected into T-deficient animals. In all probability, thymosin influences the differentiation of T-cell precursors into mature T-cells, which, in turn, normalizes the migration and differentiation of stem elements.

The utility of this line of investigations for clinical medicine remains to be elucidated. It may turn possible that some kinds of blood cancer (e.g., erythroblastosis or superfluous proliferation of red blood cells) are the result of deficiencies or impairments in the T-system of immunity. Perhaps, the dictator's function of lymphocytes with regard to stem cells is suppressed by viruses which may also cause lymphocytes to issue "wrong orders" fraught with leucoses. One thing is clear: the interaction between lymphocytes and haemopoietic stem cells is, beyond doubt, one of the main mechanisms of control over haemopoiesis.

But let us better come back to T-immunodeficiency and our experiments. Thymectomy, as we have seen, does not affect the ability of lymph node lymphocytes to direct the differentiation of haemopoietic stem cells towards myelopoiesis: T-lymphocytes of thymectomized donors do it with the same efficiency as lymph node lymphocytes of intact animals. This effect is provided for by long-living $T_2$-cells that are insensitive to thymectomy in pubescent animals. On the other hand, under conditions of T-cell deficiency resulting from thymectomy, the ability of bone marrow cells to form myeloid colonies is impaired. This ability can be restored by administration of T-lymphocytes. Now it is the activity of $T_1$-cells (but not that of $T_2$-lymphocytes) that comes to the foreground. $T_1$-lymphocytes are short-living cells that die soon after thymectomy. Does this imply that the process of stem cell maturation is controlled by more than one, to be more exact, by two T-cell subpopulations, i.e., by short- ($T_1$) and long ($T_2$)-living T-cells?

The experiments with T-deficient animals were further extended through the use of thymectomized mice as bone marrow donors and/or recipients; lethally irradiated CBA mice syngeneic to the transplanted bone marrow and lymphoid cells were used as recipients. In all cases studied, T-deficiency was concomitant with the inhibition of myelopoiesis and a shift towards erythropoiesis ($E/M \geqslant 5$). The changes in the maturation of haemopoietic precursors were especially well-pronounced upon transplantation of bone marrow cells of thymectomized donors to lethally irradiated thymectomized recipients. ($E/M = 14.5$). An addition of syngeneic lymphocytes to bone marrow

cells increased the yield of myeloid colonies and normalized the maturation of haemopoietic stem cell precursors. The E/M ratio decreased, respectively, from 5.2-14.5 to 2.9-5.9. However, neither the use of unfractionated lymph node lymphocytes, nor the addition of cortisone-resistant thymocytes to the bone marrow transplant could direct stem cell differentiation towards myelopoiesis.

Transplantation of bone marrow cells to hybrid irradiated recipients instead of to syngeneic CBA gave the same results. In thymectomized bone marrow donors and/or recipients, myelopoiesis was suppressed, while erythropoiesis predominated. However, in this case, the differentiation of stem cells, unlike that in the syngeneic donor-recipient combination, was markedly shifted towards myelopoiesis (E/M = 0.1-0.2). Cortisone-resistant thymocytes could evoke effects of comparable magnitude only when taken in much higher doses.

Thus, in T-immunodeficient animals, the normalization of differentiation of haemopoietic precursors is observed in a fully syngeneic donor-recipient system in the absence of antigenic stimulation of T-lymphocytes. This finding confirms our previously made hypothesis that the regulatory influence of T-lymphocytes on stem cells is not mediated by the GVH response. Since thymectomy in pubescent animals causes predominant elimination of short-living $T_1$-lymphocytes, it may be supposed that under normal physiological conditions the maturation of haemopoietic precursors is controlled by $T_1$-cells. Evidence for this assumption may be derived from the fact that thymectomy is concomitant with a shift in the differentiation of stem cells towards myelopoiesis.

In good agreement with these data are the results obtained by R. Khaitov and L. Ryabova (USSR) in 1978. These authors studied the maturation of stem cells in lethally irradiated mice whose bone marrow was partly protected during irradiation by lead plate screening. In old animals, 24-28 months of age, with age-specific thymus involution, the migration of stem cells from the bone marrow was suppressed; as a result, their differentiation proceeded in much the same way as that in thymectomized animals, i.e., as predominant erythropoiesis (E/M = 16). After immunization of lethally irradiated recipients with a mixture of bone marrow cells of old donors and lymphoid cells of young donors, this process was normalized, and the E/M value fell to 2.3. Presumably, old animals are deficient of T-lymphocytes which are necessary for the production of leucocytes. This defect may be overcome by transfusions of lymphoid cells obtained from young animals. In contrast with what takes place during the transplantation of bone marrow and lymphoid cells to syngeneic CBA mice, the administration of the same mixture to hybrid recipients (CBA × C57BL)$F_1$, results in predominant production of myeloid colonies. This process is controlled by $T_2$-lym-

**Table 8.** Dependence of Differentiation of Haemopoietic Stem Cells on Their Interaction with $T_D$-Lymphocytes and on Thymus Function

| Bone marrow donor (CBA) | T-lymphocyte donor (CBA) | Recipient | Predominant type of haemopoiesis in the spleen after transplantation of a cell mixture to lethally irradiated recipients | |
|---|---|---|---|---|
| | | | CBA | (CBA×C57BL)$F_1$ |
| Intact | — | Lethally irradiated | Normal | Normal |
| Intact | Intact | Ditto | Normal | Myeloid |
| Thymectomized | — | Ditto | Erythroid | Erythroid |
| Thymectomized | Intact | Ditto | Normal | Myeloid |
| Intact | — | Ditto | Erythroid | Erythroid |
| Intact | Intact | Ditto | Normal | Myeloid |
| Thymectomized | — | Ditto | Markedly erythroid | Erythroid |
| Thymectomized | Intact | Ditto | Erythroid | Myeloid |
| Intact | Thymectomized | Ditto | | Myeloid |

*Note*: Types of haemopoietic colonies: normal—E/M=2-3 : 1; erythroid — E/M = = 5-6 : 1; markedly erythroid — E/M < 10 : 1; myeloid — E/M > 1 : 1.

phocytes which are strongly challenged by hybrid recipients' antigens.

Thus, lymphocytes which control the differentiation of haemopoietic stem cells ($T_D$-lymphocytes) are present both in the $T_1$- and $T_2$-subpopulations, a feature that makes them different from T-inactivating lymphocytes which are uniquely $T_2$-cells.

The dependence of the process of differentiation of haemopoietic precursors on their interaction with $T_D$-lymphocytes as well as on thymus function is shown in Table 8. These data presented in a tabular form reflect our experimental system that simulates T-lymphocytes-induced changes in the differentiation of haemopoietic precursors in both directions. The use of T-lymphocytes permits one to repair hypererythroid haemopoiesis and even change its direction towards myelopoiesis. The latter process necessitates T-lymphocytes: under conditions of T-cell deficiency myelopoiesis is impaired.

This is the end of our story about haemopoietic stem cells and the dictator's function of lymphocytes—a coercive but, nevertheless, exigent measure aimed at the protection of the organism from foreign invaders and occasional treachery of one's own cells. Of course, the "orders", by means of which lymphocytes effect their control over haemopoiesis, may not always be sound and are, sometimes, altogether wrong. Therefore, our main task is to put the function of lymphocytes under directional control so as to be able to eliminate their errors or propel their activity in any desired direction. Works in this field are currently underway...

# Molecular Messengers of Immunity

Living organisms, the most multifarious and perfect creations of nature, are composed of a great variety of specialized systems, organs, and cells. All the constitutive elements of the organism are closely linked together and represent a strictly independent functioning whole. Starting at the origin of organs and tissues, these relationships are materialized in the course of ontogenesis, i.e., throughout the life-history of an individual.

Heart function and, accordingly, the performance of the whole cardiovascular system, are closely connected with the central nervous system, in the first place, with signals communicated by nerve cells. The heart, which is a muscular organ, rhythmically contracts and thus keeps up the circulation of the blood. For its contraction, every muscle must get an "order" from a nerve fiber. The impulse is then transmitted by the nerve ending adjacent to the muscle which ejects a special chemical substance—sends a molecular "messenger", so to speak. This substance comes into contact with a special receptor situated on the surface of the muscle cell and triggers off a chain of chemical reactions which terminate in the shortening of myosin. The latter forms the basis of myofibrils, the main components of the muscle fibre. The mechanism of muscular contraction is thus set in motion.

The major function of molecular messengers is to convey signals between different molecular structures. This is why these substances have got the name of mediators, or "go-betweens". Many cellular structures of the body consummate their functions with the help of mediators.

Mediators are concrete molecular substances of endogenous origin, i.e., they originate within the organism. Among other biological mediators discovered in the twentieth century, the most striking ones are hormones (growth hormones, sex hormones, insulin, etc.). Hormones stimulate or suppress the functions of endocrine glands and regulate the activity of many other systems of the body. Other molecular substances that are generated by the organism in the course of biochemical reactions and convey the information between different systems, or between individual organs and cells within a system, also fulfil mediator functions. Likewise, the immune system employs an army of molecular messengers whose main duty is to mediate the regulatory and effector functions of immunocompetent cells.

## Lymphocytes and Monokins

The reactions of cell-mediated and humoral immunity occurring in the organism are performed by a legion of cells, in which every subdivision is assigned to a very specialized mission. Movements of appropriate cells to the battle-ground, their instruction and fighting efficiency are provided for by the corresponding signals which are issued by "commander" cells and relaid to the neighbouring subunits. These signals, or "battle orders", come in the form of molecular substances (mediators) which serve as messengers in the immense army of immunity. The mediators secreted by lymphocytes got the name of lymphokins; those produced by monocytic cells were termed as monokins.

Let us imagine a situation when a foreign cell has entered the body. This cell is immediately encompassed by T-lymphocytes which "recognize" it by virtue of their surface receptors. T-lymphocytes signal a message to other cell populations, summoning them to receive and further convey the "orders" necessary for the most exigent extermination of the target cell. For this purpose, T-lymphocytes secrete a special mediator substance that suppresses the migration of macrophages, or MIF (from "migration inhibitory factor"). This mediator halts the macrophages flowing past the target cell and impels them to take expedient sanitary measures. In this way, the "foreigner" is rooted to the spot by T-lymphocytes and ingested by macrophages.

Effector cells can be also delivered to foreign particles by chemotaxic factors. Chemotaxis is a reaction of intense movement of cells to or back from chemical stimuli. T-lymphocytes secrete mediators which provide for positive or negative chemotaxis in other cells of the body, e.g., macrophages, neutrophils, or eosinophils. The mediators that enhance the functional activity of macrophages and lymphocytes or induce cell division are called mitogenic factors. Meanwhile, in "training schools" for "soldier" cells, the "transfer factor" translates knowledge from sensitized lymphocytes to intact "recruit" cells, i.e., those previously unexposed to the adversary.

The main objective of the attacking army, however, is the extirpation of foreign cells, and this can be achieved only with the assistance of killer cells. The reaction of killer cell formation from their precursors is mediated by the so-called interleukins. Interleukin-1, which is generated by macrophages, gives an "order" to a definite T-cell subpopulation to begin the synthesis of a T-lymphocyte growth factor, or interleukin-2. The latter plays an essential role in the stimulation and proliferation of cytotoxic T-cell precursors, or killer cells. Other accomplices in the killing of foreign target cells are lymphotoxins. These mediator proteins assist killer cells in doing away with the "foreigners" and putting under a ban their proliferation.

The molecular messengers described above participate in cell-type immune reactions mediated by sensitized T-lymphocytes as effectors. Soluble mediators can also take part in the induction of humoral immune responses aimed at the neutralization of foreign antigens with the help of antibodies circulating in the blood. In this instance, mediators yield special signals in order to stimulate definite cell populations to begin the production of antibodies.

The discovery of cell interactions at the level of induction of antibody synthesis shed new light on the mechanism of B-lymphocyte involvement in this process. Thus, it was demonstrated that besides antigens, T-lymphocytes and macrophages may also play a role in the activation of B-lymphocytes. After its exposure to the macrophage, the antigen in a strongly challenged state is delivered to T- and B-lymphocytes. Without at least one of the components of this triad, the immune reaction is impossible.

The cells of all the three types (T- and B-lymphocytes and macrophages) communicate by means of molecular mediators. The main partners of B-cells during the induction of antibody synthesis are T-lymphocytes: they manufacture the so-called helper mediator substances (aid factors) which stimulate B-cells to begin proliferating and differentiating in response to antigenic attack. As a result, B-cell precursors generate a great number of plasma cells making antibodies against foreign antigens. The major function of T-lymphocytes is the communication of signals necessary for the formation of B-cell clones. A clone is a group of cells propagated from a single precursor. The first, earliest signal is yielded in the form of an antigen-specific helper mediator which "arouses" B-cells in the case of an antigenic attack and makes them respond to the entry of foreign substances into the organism. Besides specific mediators, T-lymphocytes generate antigen-nonspecific helper factors that stimulate vigorous proliferation of B-cell clones challenged by the antigens.

Further development of B-cell clones is controlled by T-lymphocytes. Alongside helper mediators, they generate suppressor factors and thus provide for the well-coordinated propagation of B-cell clones and large-scale production of plasma cells and, correspondingly, antibodies.

Most of immunologic mediators are proteins in nature. They are heat-stable (up to 56 °C), resistant to nucleases (i.e., enzymes hydrolyzing DNA and RNA), and sensitive to proteolytic enzymes. The molecular weights of these mediators range between 10,000 and 80,000 daltons. The main physico-chemical properties of some immunologic mediators are listed in Table 9.

Besides lymphokins and monokins responsible for the induction of immune responses, there are mediators which communicate signals necessary for the normal maturation and differentiation of immunocompetent cells. Among these, the most well known are

**Table 9. Properties of Some Immunologic Mediators**

| Mediator | Functional activity | Thermostability | Susceptibility to enzymes | Chemical nature | Molecular weight, daltons |
|---|---|---|---|---|---|
| Macrophage migration inhibitory factor (MIF) | Suppresses macrophage migration | R.: 56 °C, 30 min<br>D.: 80 °C, 30 min | | Proteins | 12,000-15,000<br>50,000-80,000 |
| Transfer factor | Transfers sensitization to intact lymphocytes | R.: 56 °C, 30 min | R.: RNAase and DNAase<br>D.: pronase, xanthine oxidase | Nucleoprotein | 10,000 |
| Lymphocyte-activating factor | Stimulates proliferation of lymphocytes | | R.: trypsin<br>D.: chymotripsin, papain | Protein | 13,000-30,000 |
| Mitogenic factor | Induces blastotransformation of lymphocytes, stimulates their proliferation | R.: 56 °C, 30 min<br>D.: 70 °C, 30 min | R.: RNAase, DNAase | Protein | 20,000-30,000 |
| Nonspecific T-cell helper factor | "Assists" in activation of B-cells | R.: 56 °C, 30 min<br>D.: 60 °C, 60 min | R.: RNAase, DNAase<br>D.: pepsin, pronase | Protein | 20,000-60,000 |
| Nonspecific T-cell suppressor factor | Suppresses development of immune responses | R.: 56 °C, 30 min<br>D.: 80 °C, 30 min | R.: RNAase, DNAase<br>D.: trypsin, chymotrypsin | Glycoprotein | 48,000-67,000 |

hormones, or mediators, secreted by the thymus, one of the central organs of the immune system. These mediators contribute decisively to the development of the T-system of immunity by transmuting T-lymphocyte precursors into mature, functionally competent T-cells.

Thymectomy in mice is associated with the so-called Vasting's syndrome. Its main symptoms are the retardation of growth, baldness, intestinal disorders, changes in the blood formula, immunodeficiency (i.e., inability to reject tissue and cell grafts), lack of resistance to microbial antigens, etc. Injections of such animals with thymic extracts alleviate this condition and restore, although temporarily, the immune system. Another example is thymus hypoplasia in mice of a special strain which is associated with T-cell deficiency (i.e., lack of T-lymphocytes) and severe disturbances in the whole immune system. In such animals, T-cell deficiency can be compensated for by administration of thymus hormones. Thymic preparations exert, in addition, antitumour effect and they influence primarily the functions of the T-system of immunity. These spectacular findings fired the imagination of investigators all over the world, and the number of research teams concerned with the study of thymus hormones grew rapidly.

Not surprisingly that researchers from various laboratories who employed different methods for the isolation and characterization of thymus preparations, gave them different names. The investigator from the USA G. Goldstein succeeded in isolating an active fraction from the thymus and called it thymosin. This hormone capable of stimulating T-cell function has been extensively employed by many investigators. Attempts have been made to use this drug in pediatrics for the correction of congenital thymus hypoplasias, i.e., rare developmental diseases concomitant with partial or full T-cell deficiencies. Another US immunologist, A. Bach, isolated a thymic factor from the blood serum and termed it as FTS, i.e., factor of thymus, serumal. This author deciphered the amino acid composition of FTS and found it to be a polypeptide of nine amino acids. This discovery gave a strong impetus to the large-scale production of synthetic analogs of FTS.

Active preparations of the thymus were also obtained in a number of laboratories in the USSR. One of them—thymalin—stimulates the growth and functioning of the T-system. Experiments by V. Ya. Arion were designed to investigate the nature, biological activity, and clinical applicability of another active fraction of the thymus—AFT6, or T-activin. In terms of some physico-chemical properties and mechanism of action, this preparation has many advantages over most of the currently known thymus hormones. An extensively purified preparation of T-activin is made up of seven components. The biological significance of T-activin consists in its

ability to restore the T-system of immunity, e.g., in thymectomized animals. Its high efficiency may be illustrated by the following example. T-activin in a dose of 1 μg per 3,000,000 lymphocytes exerts the same effect as thymosin taken in a much higher dose (9-12 μg). By influencing T-cell precursors, T-activin stimulates their killer function and thus increases the T-lymphocyte population both *in vitro*—in cell cultures, and *in vivo*—in patients with various T-cell deficiencies. Preparations of T-activin have been successfully used in the therapy of some diseases of the lymphatic system, e.g., lymphogranulomatosis. It remains to be hoped that T-activin will soon find wide use as an effective means of correcting various disturbances in the T-system of immunity. Some properties of T-activin and other thymic preparations with reference to the authors who first isolated them are depicted in Table 10.

**Table 10.** Properties of Some Thymus Hormones

| Hormone | Chemical nature; mol. weight, daltons | Effects on T-lymphocytes | | | | |
|---|---|---|---|---|---|---|
| | | Expression of T-markers | Elimination of Vasting's syndrome | T-function in athymic mice | Stimulation of DNA synthesis | Antitumour effect |
| Thymosin, or fraction 5 (G. Goldstein) | Polypeptides 1,200-14,000 | + | + | + | + | + |
| Thymopoietin (G. Goldstein) | Polypeptide 5,561 | + | ? | ? | − | + |
| Factor of thymus, serumal (J. Bach) | Nonapeptide 900 | + | ? | + | − | + |
| Thymalin (Yu.I. Morozov) | Polypeptides 2,000-3,000 | ? | ? | + | ? | ? |
| T-activin, or AFT-6 (V.Ya. Arion) | Polypeptides 1,500-1,600 | + | ? | + | + | + |

Very recently, a new group of immunomediators secreted by bone marrow cells has been identified. It was found that the bone marrow which supplies the organism with stem and precursor cells, may control various immune responses of the organism. Bone marrow cells exercise their regulatory influence with the help of soluble mediators that stimulate or suppress antibody genesis. The stimulating factor of the bone marrow controls the function of mature B-cells and enhances the antibody synthesis at the peak of the immune response. This substance got the name of a stimulator of antibody producers, or SAP.

The whole complex of mediators provides for the coordinated functioning of the overall immune system; the lack of certain mediators is the cause of many pathological conditions. This is why the problem of immunomediators, besides a purely theoretical interest, has major practical implications. Many of them, e.g., thymosin, transfer factor, macrophage migration inhibitory factor (MIF), have already found wide use as valuable therapeutic and diagnostic agents.

### Classification and Nomenclature of Immunomediators

The great variety of immunomediators identified in recent years conduct differentiated functions in miscellaneous reactions of cell-mediated and humoral immunity. However, the richness of mediators, in terms of their classification and nomenclature has in the past been a considerable obstacle to analysis. One possible reason was the use, by different authors, of different test systems for the study of mediators; besides, there were no reliable criteria of their chemical structure. These and other problems have so far frustrated most attempts to isolate and purify individual immunomediators, sometimes due to objective difficulties. It is not always easy to say whether a particular function is mediated by one or several chemical substances, or whether one mediator possesses several activities.

For this reason, a great deal of effort has been invested to the improvement of the nomenclature of currently known immunomediators. A special International Committee has been set up, whose scope of competence is to elaborate rigorous criteria for the specification of mediators and unification of their nomenclature. In conformity with the project sponsored by the Committee in 1979, each mediator must be characterized in the following terms: name (full and abbreviated), test system, antigenic specificity, animal source, effector and target cells, relationship to the major histocompatibility complex, species specificity, mechanism of action, and main physico-chemical properties. Such a detailed characterization makes it possible to prove the uniqueness of a particular mediator.

The recently introduced term "interleukin" is used to describe the ability of a mediator to serve as a communication signal between different populations of immunocompetent cells. Interleukin-1 is generated by macrophages challenged by antigens or mitogens. This mediator previously termed as lymphocyte-activating factor (LAF) is a polypeptide chain with a molecular weight of 12,000-16,000 daltons. The main function of interleukin-1 is to stimulate a definite subpopulation of T-lymphocytes to begin the synthesis of T-cell growth factor, or interleukin-2. The latter also represents a single

polypeptide chain with a molecular weight of about 15,000 daltons for human and 30,000 daltons for mouse T-cells. Interleukin-2 stimulates specific receptors situated on the surface of T-lymphocytes and thus induces vigorous proliferation of T-cell clones in parallel with antigenic or mitogenic stimulation. Not long ago, another mediator of this type, interleukin-3, has been identified. Presumably, interleukin-3 affects the maturation of T-lymphocytes by inducing the synthesis of 20-α-hydroxysteroid dehydrogenase, an enzyme specific for mature T-cells. Besides, this mediator stimulates the proliferation of lymphoid cells in spleen cell cultures *in vitro*. Likewise, interleukin-3 may influence macrophages, making them synthesize interleukin-1.

Interleukins function as a single whole and thus provide for the coordinated and regulated activity of macrophages and various subpopulations of antigen-challenged T-lymphocytes. As for the classification of the currently known immunomediators, their functional activity seems to be the most reliable basic principle (Table 11).

**Table 11.** Mediators and Hormones of the Immune System

| | |
|---|---|
| 1. Humoral factors of thymus | Thymosin, thymalin, thymus factor serumal (TFS), T-activin, etc. |
| 2. Mediators providing for cell localization | Macrophage migration inhibitory factor (MIF), macrophage agglutinating factor, chemotaxic factors |
| 3. Mediators stimulating the functional activity and proliferation of cells | Transfer factor, macrophage-activating factor, lymphocyte-activating factor (interleukin-2), colony growth stimulating factor, mitogenic factor, interpherone |
| 4. Mediators suppressing the functional activity of cells | Factors suppressing colony growth, cell proliferation and DNA synthesis; lymphotoxins |
| 5. T-Cell mediators regulating antibody genesis | Specific and non-specific helper mediators; specific and non-specific suppressor mediators |
| 6. Macrophage mediators | Interleukin-1, suppressor and stimulator mediators |
| 7. Mediators of bone marrow cells | Stimulator of antibody producers (SAP), suppressor factor |

According to their functions, immunomediators can be split into 7 groups. Lymphokins and monokins (groups 2-6) mediate various interactions between T- and B-lymphocytes, macrophages, and other cells; some of them participate, side by side with lymphocytes, in

the destruction of foreign cells. The mediators assigned to groups 1 and 7 are generated by the central organs of immunity—thymus and bone marrow. These molecular messengers provide for the matura-

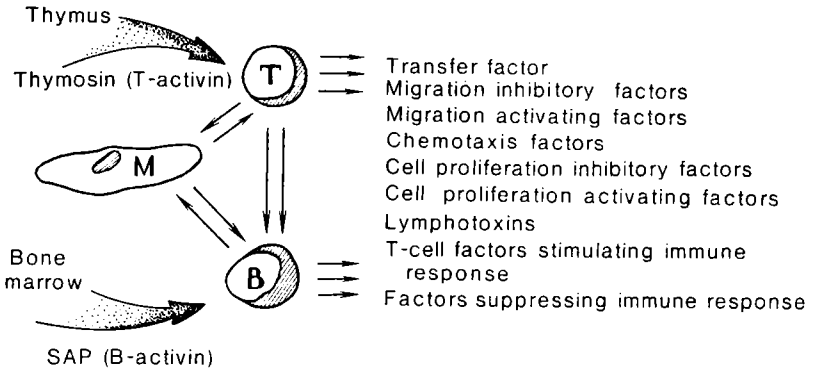



**Fig. 21.** Immunomediators expressing the relationships between various immunocompetent cells and their effector functions.
T—T-lymphocyte; B—B-lymphocyte; M—macrophage

tion and normal functioning of T- and B-lymphocytes. The interactions between the main classes of immunocompetent cells mediated by their molecular messengers are shown in Fig. 21.

## The Cells "Converse" Not Only During the Induction of the Immune Response

Numerous works dedicated to the study of cell interactions during the induction of antibody synthesis repeatedly emphasized that cell-to-cell "conversations" as well as their interrelationships and interdependencies are essential only at early stages of the immune response, when the reaction is triggered off. The signals elicited by antigens and helper T-lymphocytes are a guide to action for B-lymphocytes to begin multiplying and differentiating into plasma cells, or "antibody-producing plants". One may get an impression that once the "production lines" have been turned on, the antibody "output" will get on effectively without any outside assistance. Unfortunately, it is not quite so. Mature plasma cells need regulatory signals in order to maintain antibody genesis at an appropriate level. Therefore, the cells "converse" during the whole course of the immune reaction, in particular, at the peak of the immune response.

This fact was first established in our laboratory in 1968, in experiments with *in vitro* cultivation of lymphoid cells (Fig. 22). In this study, mice were immunized twice with an unrelated protein (antigen). Four days after the second immunization, the lymph nodes

and spleens of all experimental animals appeared to contain a large number of plasma cells making antibodies against the immunizing antigen. The peak of the immune response was about to follow. At this point, lymph node cells were removed and placed into *in vitro* cultures. The transfer procedure did not influence the viability or functional activity of the lymphocytes. After 18 hours, the number of antibody-secreting cells and that of the antibodies formed were determined; the results were designated as $N_1$ and $N_2$. In parallel

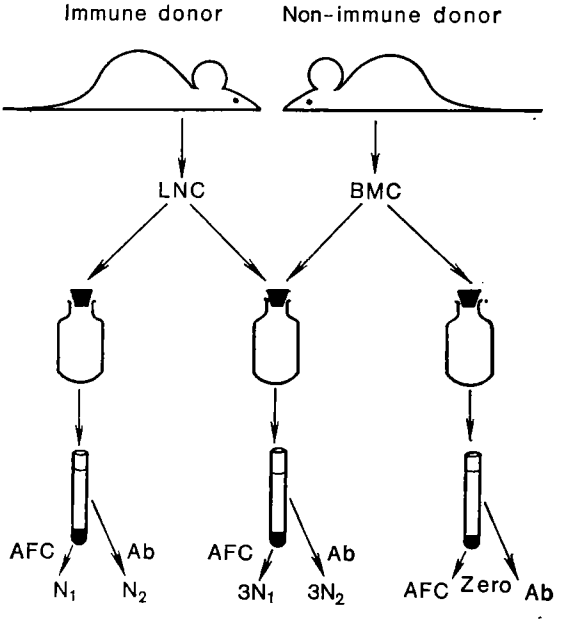



**Fig. 22.** Study of cell cooperativity at the level of mature antibody producers (diagram of principles).

**LNC—lymph node cells; BMC—bone marrow cells; AFC—antibody-forming cells; Ab—antibodies**

experiments, lymph node cells of the same mice were placed into culture media, to which equal amounts of bone marrow cells of intact (non-immunized) mice were added. The number of antibody-producing cells and antibodies reassessed 18 hours later appeared to be three times as great, i.e., $3N_1$ and $3N_2$. A 3-fold increase in the number of "plants" and their specific antibody "output". It is noteworthy that the level of non-immunoglobulin proteins in the mixture was not so high as that observed during separate cultivation of cells (Fig. 23). Hence, in mixed cell cultures, the interactions between cells provided for the augmented synthesis of antibodies.

No enhancement of antibody production was observed, however,

when lymph node cells of immunized mice were mixed with renal, liver, or thymus cells of non-immunized animals. The cells with the ability to stimulate mature antibody producers and make them

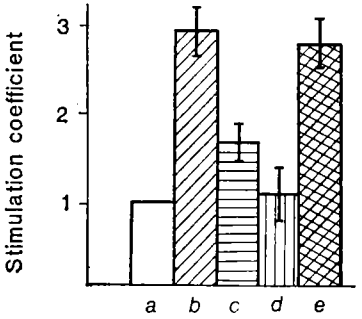



**Fig. 23.** Stimulation of antibody synthesis in a mixed cell culture composed of lymph node lymphocytes of immune mice and bone marrow cells of intact mice:

*a*—monoculture (control); *b*—antibody synthesis in a mixed culture; *c*—non-specific immunoglobulin synthesis; *d*—non-immunoglobulin protein synthesis; *e*—number of antibody-forming cells

synthesize increasing amounts of antibodies were localized exclusively in the bone marrow. We termed this new, previously unknown type of cell interactions as "cooperation at the level of mature antibody producers".

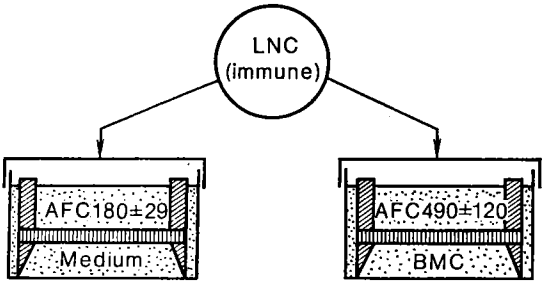



**Fig. 24.** Stimulating effect of bone marrow cells on antibody synthesis in the population of mature antibody producers during cultivation of cells separated by a millipore membrane.

LNC—lymph node cells; AFC—antibody-forming cells; BMC—bone marrow cells

What are the events that take place during the interaction of bone marrow cells with mature antibody producers? Which of them "give orders" and which "submit" to them? Perhaps, some of B-cell precursors present in the bone marrow begin to secrete antibodies at the very first contact with the immune population. Or, conversely,

the bone marrow "orders" the antibody producers to increase the antibody "output". Solutions to these problems came from the use of an experimental system elaborated by L. A. Zakharova (USSR). This system is based on the cultivation of immunocompetent lymph node lymphocytes and bone marrow cells on both sides of a millipore membrane that is penetrable for solute cell components but impenetrable for cells. After 18 hours, the number of antibody-forming cells was calculated in both cell populations. Whereas among bone marrow cells the antibody producers were absent, in the lymph node population their number increased 3-fold (Fig. 24). So it is bone marrow cells that "order" mature antibody producers to increase in number and augment the antibody synthesis. Apparently, the bone marrow comprises certain cells which control the intensity of antibody formation at the peak of the immune response. Studies were initiated in order to find out how these mechanisms operate...

### SAP Is a Messenger of Stimulator Bone Marrow Cells

Experiments with the use of millipore membranes led us to another important observation. Bone marrow stimulates the antibody synthesis in immunocompetent cells indirectly, without coming into contact with them. Bone marrow cells appear to secrete a soluble factor (mediator) which provides for the 3-fold increase in the number of mature antibody producers in the immune population. This mediator got the name of a stimulator of antibody producers (SAP).

SAP which mediates cell interactions at the level of mature antibody producers would be expected to accumulate in the medium during the cultivation of bone marrow cells. In order to test this possibility, lymph node lymphocytes of immune animals were added to the culture fluid, in which bone marrow cells had been kept for 18 hours. The stimulating effect of SAP on antibody synthesis reproduced with the same yield, i.e., $3N_1$ and $3N_2$! These experiments confirmed the hypothesis suggesting that SAP is a product of normal metabolism of bone marrow cells which does not require any exogenous stimuli for its synthesis. SAP is accumulated in the culture medium containing bone marrow cells in the absence of antigens or mitogens, i.e., substances that stimulate cell division.

SAP is more than a mere product of cell degradation that is excreted into the environment from the intracellular space; it is synthesized by living, actively metabolizing cells. Bone marrow cells disrupted by homogenization or a freezing-thawing procedure lose their ability to produce SAP. Accordingly, SAP is a product of *de novo* synthesis; it is accumulated in the medium within the first six hours after the beginning of cultivation and is pooled there as long as the cells

are alive and functioning. With cell decay, SAP synthesis momentarily comes to a halt.

The next problem that arose on our way was the isolation and purification of SAP from other components present in the culture medium. This measure is exigent because all of the products of cell metabolism are excreted into the environment. Besides, every culture medium contains various nutrients necessary for the normal functioning of cells, e.g., amino acids, vitamins, proteins, etc. For the isolation and purification of SAP, we employed the method of gel filtration on Sephadexes.

Sephadexes consist of spherical particles, or granules, perforated with tiny pores which, like molecular sieves, sort out substances

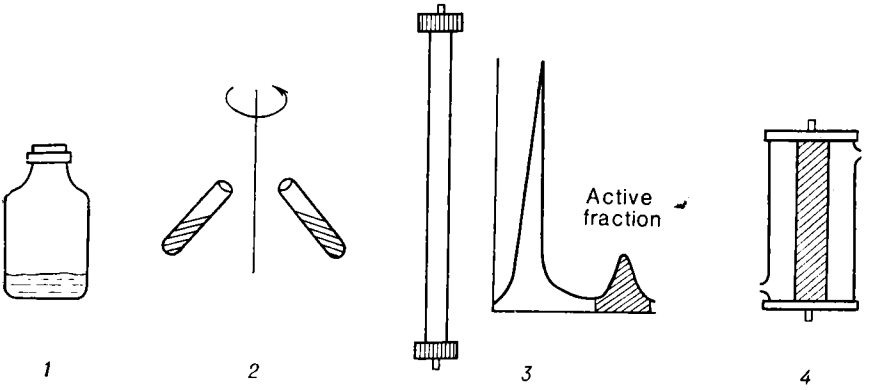



Fig. 25. Isolation of SAP from the supernatant of bone marrow cell culture:
*1*—cultivation of cells for 18-20 hours; *2*—sedimentation of cells by centrifugation; *3*—gel filtration of supernatant on Sephadex; *4*—concentration of the active fraction

according to their molecular size. During filtration of a mixture of substances through a glass column packed with Sephadex, large molecules that cannot penetrate inside the pores, pass between Sephadex granules and therefore move faster down the column. Small molecules that can pass into the pores, flow more slowly. In this way, molecules of different sizes are eluted from the Sephadex column in the order corresponding to the decrease of their molecular weights.

Aliquots of samples collected in the eluate were added to immune lymph node lymphocytes for the activity assay. The most active fraction was separated, concentrated to a required volume, and assayed for protein content. The mediator dose was adjusted correspondingly. The resulting preparation of SAP was used in all further analyses. The procedure of isolation of SAP from murine bone marrow cells is schematized in Fig. 25.

## Molecular Nature of SAP

Using gel filtration on Sephadexes, we determined the molecular weight of SAP. On Sephadex G-100 whose pore diameter allows the passage of large molecules, the SAP-containing fraction was eluted from the column with an elution pattern similar to that of cytochrome *c* (mol. weight 13,000 daltons). Upon filtration of the same fraction on Sephadex G-15 having a smaller diameter of pores, the molecular weight of SAP could be determined with a greater degree of accuracy; in this case, SAP was eluted from the column as three active fractions with molecular weights ranging between 1,300 and 5,000 daltons.

SAP appeared to be a thermostable compound which retained its biological activity upon heating to 80 °C for 30 min and even to 100 °C for 10 min. The mediator is made up of two components, one peptide and one ribonucleic.

Evidence for the dual nature of SAP can be derived from the following experiments. When radioactive (e.g., $^{14}$C-labelled) uridine or glycine is added to a medium containing bone marrow cells, the label will incorporate into the compounds synthesized by these cells in the course of their cultivation. In our studies, the $^{14}$C-label was detected within the composition of SAP. Under the stipulation that uridine is specific for RNA, and glycine is a constituent of proteins, SAP would be expected to have a ribonucleoprotein origin, i.e., to contain both RNA and protein. This hypothesis was confirmed in experiments with RNA- and protein-hydrolyzing enzymes. Treatment of SAP with protease A, pronase, or RNAase eliminated the stimulating effect of the mediator on antibody formation.

Besides, we studied the molecular nature of SAP, using the inhibitory analysis. Bone marrow cells were incubated for 3 hours with inhibitors of DNA, RNA, and protein synthesis. The cells were then washed to remove traces of the inhibitors and transferred to culture media, in which their ability to produce SAP was tested. The results of this analysis demonstrated that SAP needs RNA and protein (but not DNA) for its synthesis.

Thus, SAP can be defined as a low molecular weight, thermostable, RNA-containing peptide. For a greater accuracy of determination of the molecular structure of SAP, the mediator has to be purified to homogeneity.

In our experiments, the SAP-containing fraction was separated into constituent components by isoelectrofocusing on ampholines and electrophoresis in polyacrylamide gel. These procedures resulted in six differently charged active subfractions, all of which could effectively stimulate the antibody synthesis.

It seems very likely that SAP, like many other mediators (e.g., thymus hormones), is an association of homologous molecules.

## Properties of SAP-Producing Cells

Structurally, the bone marrow is a very heterogeneous organ. Besides large cell populations, such as lymphocytes, granulocytes and erythrocytes, it contains small quantities of thrombocytes, eosinophils, basophils, and mast cells. It also includes monocytes and macrophages. Which of them synthesize SAP? To clarify this question, we used different methodological approaches, in

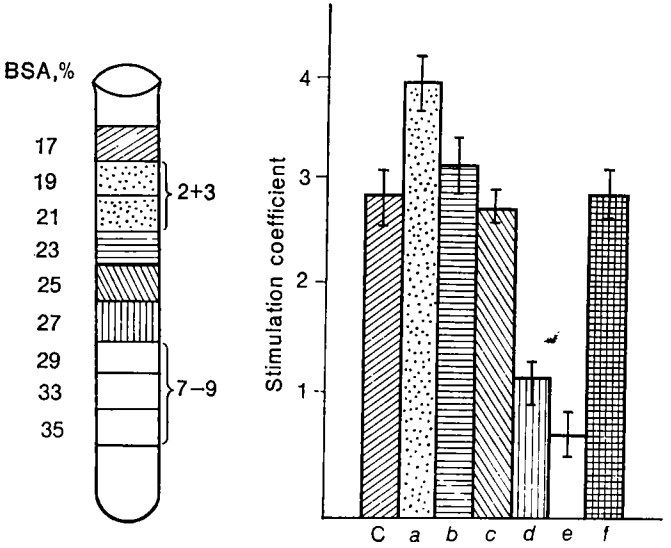



**Fig. 26.** Stimulating effects of various fractions of bone marrow cells obtained by centrifugation in the density gradient of bovine serum albumin (BSA):

C—control; *a*—fraction 2 + 3; *b*—fraction 4; *c*—fraction 5; *d*—fraction 6; *e*—fraction 7 + 9; *f*—combined fraction

particular, centrifugation of bone marrow cell suspensions in the density gradient of bovine serum albumin (BSA).

Solutions of BSA in decreasing concentrations were layered on top of one another in centrifuge tubes, so that the topmost layer corresponded to the protein with the lowest concentration. Bone marrow cells were placed over the preformed gradient. In the course of centrifugation, the cells sedimented according to their density: heavy cells penetrated inside the dense layers of BSA, while light ones accumulated in the upper layers of the gradient. After termination of centrifugation, the fractions of bone marrow cells were washed to remove traces of BSA and added to the cultures of immune lymph node cells for estimation of the SAP-producing ability.

The cells with the ability to stimulate antibody genesis appeared to be accumulated in the light layers of the gradient (Fig. 26).

Fractions 2 and 3 possessed the highest activity; fraction 1 largely composed of cell fragments was not used in the assay. The activity of fractions 4 and 5 was close to that of unfractionated bone marrow cells. Heavy fractions (6 to 9) contained suppressor cells. The stimulating activity of combined fractions was comparable to that of the original suspension. As can be evidenced from these experiments, the fractionation procedure had no effect on the stimulator properties of bone marrow cells. Another important finding was that SAP-producing cells have a low specific density—a feature which makes them similar to less differentiated juvenile cells. In another experimental series, we studied the sensitivity of SAP-producing cells to ionizing radiation.

Bone marrow cells of intact mice were transferred to culture media and irradiated with $\gamma$-$^{60}$Co rays. After irradiation, the cells were washed and then cultivated together with lymph node lymphocytes of immune animals, after which their ability to stimulate the antibody synthesis was estimated. It appeared that irradiation of bone marrow cells with doses as high as 20,000 R did not eliminate their stimulator activity (Table 12). Presumably, SAP-producing cells are highly radioresistant.

**Table 12.** Effects of Various Treatments of Bone Marrow Cells (BMC) on Stimulation of Antibody Synthesis in Immune Lymph Node Cell (LNC) Cultures

| Treatment of BMC | Number of AFC, $\times 10^6$ | | | | Stimulation coefficient | |
|---|---|---|---|---|---|---|
| | control group | | experimental group | | control group | experimental group |
| | LNC | LNC + BMC | LNC | LNC + BMC | | |
| Mice ("nude") | 56±6.0 | 148±5.6 | 63±6.6 | 176±25.9 | 2.54 | 2.81 |
| Anti-Thy-1,2 serum + complement | 69±14.9 | 126±15.0 | 69±14.9 | 122.5±8.0 | 1.83 | 1.78 |
| Anti-Ig serum + + complement | 73±8.0 | 216±13.2 | 73±8.0 | 200±14.1 | 2.96 | 2.74 |
| Passage of cells through a columnfilled with glass wool | 55±3.0 | 116±2.0 | 55±3.0 | 52±5.5 | 2.11 | 0.95 |
| Treatment with latex | 39±4.6 | 73±3.1 | 39±4.6 | 68±3.5 | 1.87 | 1.74 |
| Irradiation (20,000 R) | 48±5.1 | 114±4.6 | 51±3.0 | 112±3.8 | 2.38 | 2.2 |

*Note*: AFC — antibody-forming cells

A specific feature of functionally heterogeneous cells is the presence on their surface of specialized macromolecular identifying factors (markers). Antibodies directed against certain surface markers specifically bind to the cells "labelled" with a particular marker. Treatment of "labelled" cells with special serumal substances (complement) causes cell lysis, i.e., destruction. In this way, one can remove certain cell populations from the mixture. We used this technique in order to evaluate the contribution of T- and B-lymphocytes to SAP production.

A suspension of bone marrow cells was treated with antisera containing antibodies against antigen Thy-1,2 or immunoglobulin (Ig), specific markers for T- and B-lymphocytes, and complement. Such treatment allowed us to subtract the Thy-1,2 and Ig-labelled cells from the suspension.

An analysis of the stimulating activity of antisera-treated bone marrow cells revealed that SAP synthesis occurs independently of T- and B-lymphocytes. The stimulation of antibody synthesis was observed after treatment of bone marrow cells with anti-Thy-1,2 or anti-Ig sera and complement (Table 12). Further evidence for SAP production in the absence of T-lymphocytes was obtained in our experiments with athymic mice of the "nude" strain. These animals have congenital thymus hypoplasia associated with a lack of mature, functionally active T-cells. Bone marrow cells of "nude" mice added to lymph node lymphocytes appeared to stimulate the antibody synthesis in much the same way as did the bone marrow cells of healthy mice in the control group (Table 12).

We also examined the ability of SAP-producing cells to adhere to glass and phagocytize latex. Monocytic cells (especially macrophages) are strongly adhesive and are therefore called sometimes A-cells ("A" stands for "adhere"). During filtration of bone marrow cells through a column filled with glass wool, some of them were retarded being adhered to glass particles; the cells eluted from the column were mixed with mature antibody-forming cells (immune lymph node lymphocytes). The removal of cells adhered to glass wool appeared to eliminate the stimulating effect of bone marrow cells (Table 12). At the same time, SAP-producing cells did not possess the phagocyting activity. During incubation with latex (37 °C, 1 hour), the phagocyting cells of the bone marrow ingested latex particles, but their functional activity did not change, as would be expected; the blocking of phagocytes in the bone marrow cell suspension did not practically affect the value of the stimulation coefficient (Table 12).

Hence, SAP-producing cells cannot be related either to the T- or B-lymphocyte populations. These cells adhere to glass wool and are radioresistant. In all probability, SAP producers are monocytic cells, most likely, juvenile forms of promonocytes.

## SAP Makes Reserve "Silent" Cells "Talk"

How does SAP increase the number of antibody-forming cells at the peak of the immune response? What is the mechanism of the stimulating effect of this mediator? Answers to these questions may come from the analysis of events that take place during the antibody synthesis.

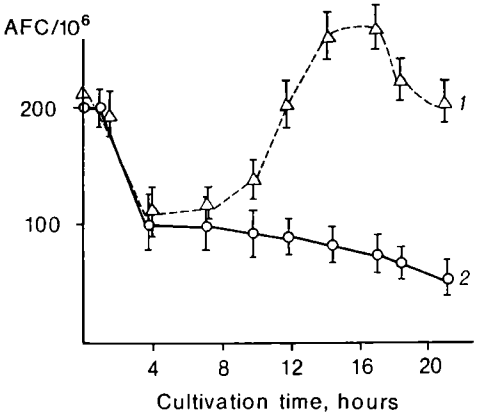
SAP may increase the number of antibody-forming cells in the immune population in two ways: either by stimulating their proliferation or by involving additional, non-proliferating cells in antibody genesis. It thus seemed reasonable to investigate the role of cell division in the stimulation of antibody synthesis at the peak of the immune response. If one follows the accumulation of antibody-forming cells in the immune population through time, the stimulating effect will be observed already 6 hours after the beginning of cultivation and reach the maximum by the 10-15th hour (Fig. 27). Such an early manifestation of the stimulator activity of SAP may indicate that the appearance of antibody-forming cells in the population of mature antibody producers is not the result of cell division. Direct evidence for this assumption was obtained in the following experiments.

Lymph node cells of immune mice were cultivated in the presence or absence of bone marrow cells. To both cultures, $^{14}$C-thymidine was added. Cell division is generally accompanied by the synthesis of DNA whose molecule incorporates thymidine. Thus, the role of cell proliferation can be determined from the amount of the radioactive label incorporated into DNA.

In our studies, an addition of bone marrow cells to culture media containing mature antibody producers did not change the rate of their proliferation. At the same time, under effects of SAP, the number of antibody-forming cells increased 2- or 3-fold, thus indicating that the accumulation of antibody producers in the immune population is not coupled with cell division. Similar results were obtained in experiments with the use of inhibitors of DNA synthesis. Immune lymph node cells were treated with 5-bromodeoxyuridine or mitomycin C which suppressed the DNA synthesis and blocked cell proliferation. The treated cells were mixed with intact bone marrow cells; under these conditions, the stimulating effect of SAP on antibody genesis persisted irrespective of blocking of DNA synthesis. This may be regarded as an additional proof of the lack of correlation between the synthesis of antibody-forming cells and cell division.

These observations led us to suppose that in the productive phase of antibody synthesis, the population of mature antibody producers contains reserve "silent" cells, in which the antibody genesis is blocked. SAP stimulates this process, thus providing for a consider-

able enlargement of the antibody producer population at the peak of the immune response. The involvement of "silent" cells in antibody synthesis at the peak of the immune response without cell division was demonstrated by many authors on a great variety of experimental models and test systems. In one, the suppression of cell proliferation by colchicine strongly influenced the number of antibody-forming spleen cells removed at early stages of the immune response. No



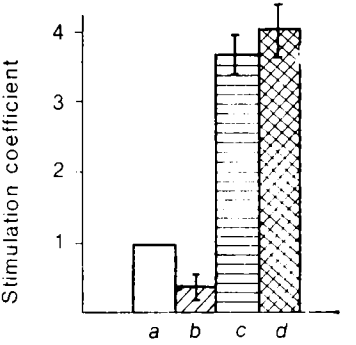




**Fig. 27.** Stimulation of antibody synthesis in a mixed culture of lymph node cells of immune mice and bone marrow cells of intact mice:
*1*—monoculture; *2*—mixed culture

**Fig. 28.** Stimulation of antibody synthesis after addition of bone marrow cells and T-suppressors to an immune lymph node cell culture:
*a*—monoculture (control); *b*—immune lymph node cells + T-suppressors; *c*—immune lymph node cells + bone marrow; *d*—immune lymph node cells + bone marrow + T-suppressors

such effect was observed in *in vitro* cultures, when spleen cells were obtained from the donors on the 4th day of the secondary immune response, i.e., at the peak of the immune reaction.

In another model experiment, splenic B-cells of mice twice immunized with sheep erythrocytes secreted a humoral factor capable of increasing the number of antibody-forming cells in the population of mature antibody producers. After addition of this factor to a culture of spleen cells removed on the 5th day after the first immunization, the number of antibody producers increased 2-fold 2 hours after the incubation with the factor. These data also point to possible involvement of resting cells in antibody synthesis unaccompanied by cell division.

It follows, therefore, that at the peak of the immune response, the immune population, besides active antibody producers, contains a certain amount of reserve cells ready to begin the antibody synthesis.

Such cells provide for additional regulation of antibody synthesis at the peak of the immune response.

The mechanisms of suppression of antibody genesis in "silent" cells are currently obscure. In all probability, this process is mediated by suppressor T-lymphocytes which "prohibit" mature antibody producers to secrete antibodies. As we have seen in the preceding sections, T-lymphocytes may sometimes control various reactions of humoral immunity. Certain T-lymphocyte subpopulations generate helper or suppressor mediators that stimulate or suppress the proliferation of B-cells in response to antigenic stimulation. SAP, which eliminates the T-cell-induced suppression of antibody synthesis at the peak of the immune response, increases the number of antibody-forming cells and thus stimulates antibody genesis.

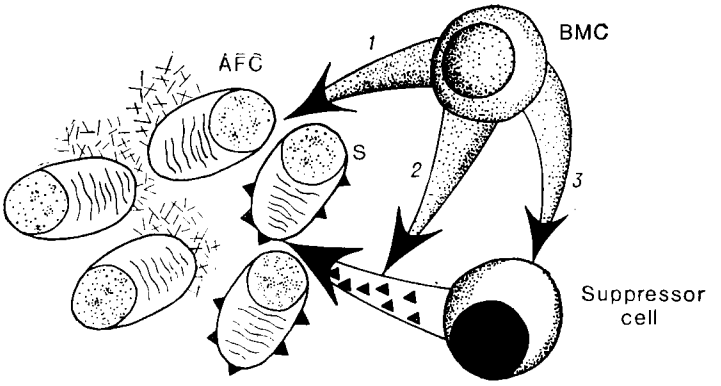



Fig. 29. Possible directions of the stimulating effects of SAP
AFC—antibody-forming cells; BMC—bone marrow cell; S—'silent' cell; *1*—elimination of suppression of 'silent' cell; *2*—neutralization of the suppressing factor; *3*—blocking of activity of suppressor cells

In order to clarify this problem, we studied combined effects of T-suppressors and bone marrow cells on antibody synthesis in the productive phase of the immune response.

T-suppressors were obtained by *in vitro* cultivation of antigen-challenged murine T-lymphocytes in the presence of the same antigen. The T-suppressors so produced were added to lymph node cells obtained from the donors on the 4th day of the secondary immune response. After incubation for 15 hours, the number of antibody-forming cells was determined in the cultures.

The suppression of antibody genesis by T-lymphocytes at the peak of the immune response appeared to be increased 3-5-fold (Fig. 27). Apparently, T-suppressors can block the antibody synthesis in the productive phase of the immune response as well. In other experiments, bone marrow cells were added to immune lymph node lymphocytes simultaneously with T-suppressors. In such mixtures,

the stimulation of antibody genesis appeared to be the same as that in the absence of T-suppressors (Fig. 28).

Thus, in the presence of bone marrow cells, T-suppressors are "passive". It seems probable that SAP eliminates the suppressor activity of T-lymphocytes.

The mechanisms of action of SAP on the alleviation of suppression of "silent" cells are still unclear. SAP may influence the suppressed cells directly, or block the suppressor factor or its precursor. Possible competitive interactions of SAP with suppressor T-lymphocytes are shown in Fig. 29.

All these data illustrate the ability of SAP to make "silent" cells "talk" by relieving them of the suppressive influence caused by T-lymphocytes. Presumably, this mediator controls the antibody synthesis at its ultimate stage, i.e., at the level of mature antibody producers. Under conditions of antibody deficiency, the stimulating effect of SAP on reserve antibody-forming cells is a feed-back one. This new, previously unknown regulatory mechanism may be regarded as a reserve pathway of control over antibody genesis which is directed against foreign antigens entering the organism.

## SAP Is Active Both in Vitro and in Vivo

The activity of most of the currently known immunologic mediators is assessed in various model systems based on the use of cell cultivation *in vitro*. Thus, the activity of MIF is measured by changes in the square occupied by target cells in flat-bottomed grooves or upon their discharge from a capillary. The activity of chemotaxic factors is determined by an increase or decrease in the concentration of cells placed into a special chamber containing the tested substance, to which the mediator is then added.

Lymphotoxins are recognized by their cytotoxicity in *in vitro* cultures. But very few mediators (e.g., humoral factors of thymus and transfer factor) display their activity *in vivo*, i.e., within a human or animal organism. The development of techniques for identifying mediator activities within the context of the whole organism, i.e., in an *in vivo* system, seems to be of great theoretical and practical importance, for it provides guide-lines for the use of immunomediators in the correction of various immunodeficiencies.

In model experiments *in vivo*, we studied the effects of SAP injected into the footpad of one of the hind limbs of mouse on the cells of adjacent lymph nodes. Prior to SAP injections, the animals were twice immunized with an antigen (sheep erythrocytes). The peak of the immune response was observed on the 4th day after the second injection. By that time, mature plasma cells making antibodies against sheep erythrocytes began to accumulate in large amounts in peripheral lymphoid organs (lymph nodes and spleen). The ani-

mals were then given a second dose of SAP that was injected into the same footpad; the footpad of the other hind limb (control) was injected with an isotonic solution of NaCl. On the next day, the

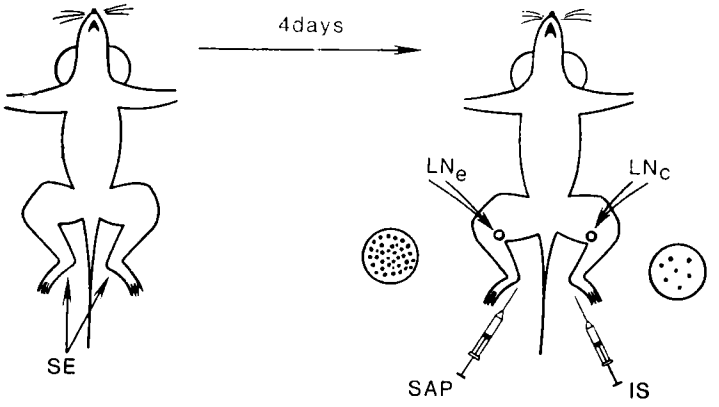



**Fig. 30.** Estimation of the stimulating activity of SAP after its local injection into the footpad of a hind limb of an immune mouse:
SE—sheep erythrocytes; $LN_e$ and $LN_c$—lymph nodes of experimental and control limbs respectively; IS—isotonic solution of NaCl

number of antibody-forming cells was estimated in the adjacent popliteal lymph nodes of both control and experimental limbs. It appeared that after SAP injections the number of antibody producers

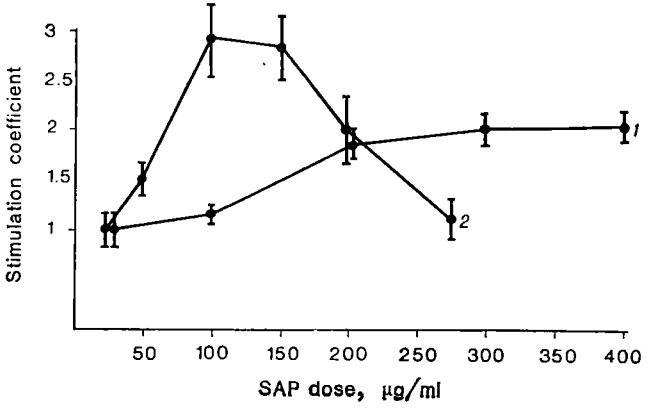



**Fig. 31.** Dose-dependent stimulating effects of SAP *in vitro* (*1*) and *in vivo* (*2*).

in the adjacent lymph nodes increased 2-3-fold as compared with the control—and again the same ratio of N and 3N! Hence, the stimulating effect of SAP on antibody genesis is observed not only *in vitro*, but also *in vivo*, i.e., within the living organism (Fig. 30).

This model proved to be very convenient for rapid quantification of SAP effects in various *in vivo* systems. Its main advantage is that it allows a comparison of the number of antibody-forming cells in two different lymph nodes of the same animal and thus provides for the great accuracy and high reproducibility of experimental results.

The activity of SAP *in vivo*, however, is not confined to its use for local injections. When this mediator is administered intravenously or subcutaneously at the peak of the immune response, it increases the number of antibody-producing cells in all of the lymph nodes of experimental animals. The stimulating effect of SAP on antibody synthesis is dose-dependent. The optimal dose of the mediator in an *in vitro* system ranges between 50 and 70 μg/ml; that in an *in vivo* system—between 100 and 200 μg/ml per animal (Fig. 31).

## SAP Is Produced in the Bone Marrow of Various Animal Classes and Species

The high potentialities of SAP *in vivo* raised the problem of its clinical utilization. That would be possible, however, only if SAP obtained from animals of one genotype could stimulate the antibody synthesis in animals of other species.

The cooperative relationships between T- and B-systems of immunity are realized only under conditions of genetic identity (syngeneity) of T- and B-cells. Unrelated T-lymphocytes are incapable of stimulating B-cells to begin the antibody synthesis. Actually, the practical utility of this cooperation is next to impossible, for the absolute majority of diseased individuals have no genetically identical twin sisters or brothers. On the other hand, non-syngeneic T-lymphocytes and the mediators secreted by them cannot stimulate B-cells. SAP appeared to be particularly well-suited for the purpose. Bone marrow cells obtained from animals of different classes and species were added to mature antibody-forming cells of immune mice. The samples containing antibody producers mixed with syngeneic bone marrow cells served as controls (Fig. 32). Stimulation of antibody genesis in the genetically heterogenous cell mixtures was essentially the same as that in control samples. SAP is known to be produced by bone marrow cells of both animals and humans; its stimulating effect on antibody genesis is not species-specific. This circumstance provides a way for SAP production from bone marrow cells of any animal species as well as for its further use for immunocorrections in both animals and humans.

This remarkable property of SAP suggests it to be of great use for pharmacologists. This compound which is endogenous in nature

exerts minimal adverse side-effects when injected into the organism with therapeutic purposes. Tremendous potentials of SAP stimulate

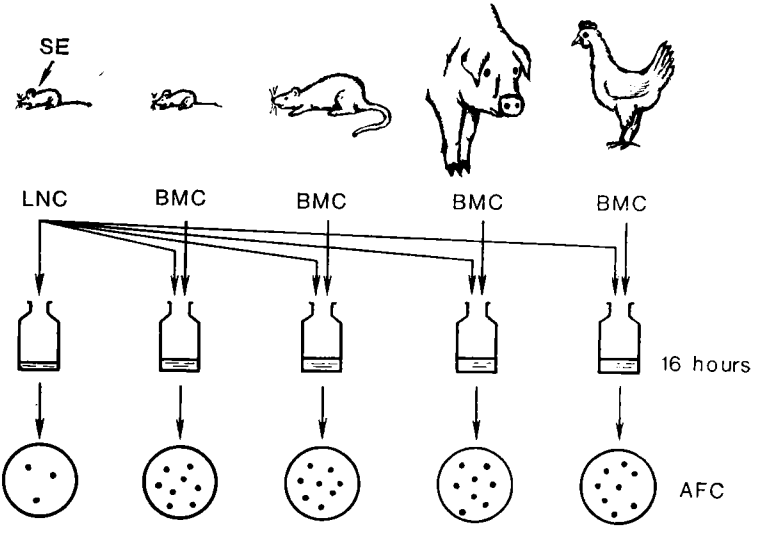



**Fig. 32.** Study of species-specificity of SAP.
SE—sheep erythrocytes; BMC—bone marrow cells; LNC—lymph node cells; AFC—antibody-forming cells

researchers to look for new ways and means of practical utilization of this mediator in clinical and veterinary medicine.

## SAP Helps the Organism to Combat Infections

The usual practice in the therapy of some infectious diseases is the injection of patients with γ-globulins, i.e., antibody preparations capable of neutralizing the toxic effects of microbes. Yet, a far more effective and natural way of enhancing the antibody synthesis in the injured organism is the mobilization of one's own protective resources. Will SAP be of any help in this situation? Can this mediator endowed with the ability to make "silent" cells "talk" help the organism to combat infections, amplify the effects of vaccines, and correct various B-cell deficiencies? Perhaps, SAP can be used in the production of therapeutic and diagnostic antisera with high antibody titers or become a helpful tool in the therapy of various allergic diseases, for one of the main causes of allergy is the inability of the organism to secrete antibodies against various allergens. With this in mind, we developed a number of experimental models stipulating practical applications of SAP. One of such models is encephalitis in mice.

Mice virally infected with Japanese and tick-borne encephalitis were injected with SAP (50 μg per animal), once (on the 8th day) or thrice (on the 3rd, 5th, and 8th days) after the infection. Control animals received injections of an isotonic solution of NaCl, instead of SAP. On the 10th day, the antibody titers were determined in the blood sera of control and experimental animals.

In animals infected with tick-borne encephalitis and injected with a single dose of SAP on the 8th day, the antibody titers increased 4-fold, i.e., from 1 : 10 to 1 : 40. In animals infected with Japanese encephalitis, the stimulating effect of SAP appeared to be twice as great: the number of antibodies increased 8-fold—from 1 : 5 to 1 : 40. In the other experimental group, in which the animals were injected with SAP on the 3rd, 5th, and 8th days (total SAP dose—150 μg per animal), the increase was 6-fold, i.e., from 1 : 10 to 1 : 60 in the case of Japanese encephalitis and 16-fold (from 1 : 10 to 1 : 160) in the case of tick-borne encephalitis.

It appears, therefore, that in acute infections such as the ones we have just witnessed in arbovirus-infected animals, SAP stimulates the antibody synthesis and increases the antibody titers in the blood sera. Similar effects are observed in the case of chronic infections, e.g., in experiments with mice infected with $10^2$ $LD_{50}$ of Japanese encephalitis virus.

Apart from stimulating the antibody synthesis in encephalitis-infected animals, SAP increased their survival rate. A group of mice infected with Japanese encephalitis virus ($10^5$ $LD_{50}$) was followed up over a period of two months. On the 4th day after infection, the animals were injected with SAP (50 μg per animal). Under effects of SAP, the survival rate increased almost 2-fold! The results assessed after the period of two months demonstrated that the mortality in the experimental group was only 5% as compared to the 50% in the control group.

In a special series of experiments, we studied combinatorial effects of SAP and some thymic preparations on antibody genesis. Under the stipulation that SAP enhances the activity of B-cells and thymic factors provide for the normal maturation and functioning of T-lymphocytes, their combined injections would be expected to be beneficial for the organism, the more so that a humoral response normally involves the cells of both types, i.e., T- and B-lymphocytes. The former help the latter to ultimately evolve into mature antibody-producing cells.

Thymic factors were subcutaneously injected into mice in two doses, prior to their first immunization with sheep erythrocytes. SAP was injected subcutaneously at the peak of the secondary immune response. On the 5th day after the second immunization, the animals were sacrificed, and the number of antibody-forming cells in their lymph nodes was determined.

The effects of these preparations appeared to be additive. When injected separately, T-activin and SAP increased the antibody synthesis in the lymph nodes approximately 2-fold, whereas after their combined injection the stimulating effect was 5 times as great. These data aptly illustrate the utility of both drugs for the correction of various immunodeficiencies.

The therapeutic value of SAP is all the more high, because the adverse side-effects of this endogeneous compound are minimized. Clinical trials have demonstrated that SAP is a non-toxic, non-allergic substance which has no influence on the genetic system of the cell. This particular feature makes SAP a useful mould for the creation of new medicinal agents to be used in clinical and veterinary medicine.

## Suppressor Mediators of the Bone Marrow

The discovery of the stimulating effect of bone marrow cells on antibody genesis shed new light on the role of bone marrow in various reactions of humoral immunity. From this standpoint, the bone marrow is not only a forge of B-cell precursors, but also as a source of cells that control the induction and development of immune responses. Soon after the discovery of SAP, several soluble mediator substances with the ability to suppress antibody genesis were identified.

The suppressor activity of bone marrow cells was first detected in experiments with transplantation of bone marrow cells in conjunction with antigen (sheep erythrocytes) to mice of CBA and C57BL strains differing in their responsiveness to this particular antigen. Immunization of the high responder CBA mice with a bone marrow—antigen mixture led to the suppression of antibody synthesis, while in the case of the low responder C57BL strain the bone marrow cells stimulated the immune response. This finding led us to the assumption that the observed differences may be due to the unequal ratios of the interacting cells in different recipients. This hypothesis was confirmed by experiments conducted by R. Ataullakhanov (USSR).

Spleen cells ($2.5 \cdot 10^6$ per 1 ml) were cultivated in the presence of sheep erythrocytes. The number of antibody-forming cells estimated on the 4th day, ranged between 300 and 2,000 per every million of cells, depending on experimental conditions. After simultaneous addition of bone marrow cells and sheep erythrocytes to spleen cell cultures, the number of antibody-forming cells decreased sharply (8-10 times). The suppressor effect was maximal when the number of bone marrow cells added to the culture was equal to that of spleen cells. With a decrease in the relative number of bone marrow cells, the suppression was either eliminated or altogether absent. The

suppressor effect was absent at the spleen to bone marrow cell ratios of 6-8 : 1.

Studies with cultivation of spleen and bone marrow cells on both sides of a millipore membrane in a special two-chamber vessel demonstrated that the suppression of antibody synthesis is mediated by a soluble factor of bone marrow origin. During separate cultivation of these cells, the immune response of spleen cells to sheep erythrocytes was stimulated (Fig. 33). However, after addition of spleen

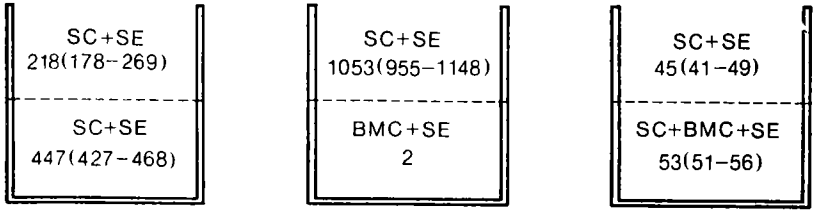



Fig. 33. Detection of a suppressor mediator of the bone marrow. The numeric values designate the number of antibody-forming cells (AFC) per $10^6$ viable cells during cultivation of spleen (SC) and bone marrow (BMC) cells in the upper and lower chambers partitioned by a millipore membrane. SE—sheep erythrocytes

cells to the chamber containing bone marrow cells, the immune response appeared to be suppressed in both chambers. The exposure of splenocytes to bone marrow cells induced the synthesis of a bone marrow suppressor factor which penetrated across the millipore membrane and suppressed the immune response in the spleen cell population.

The ability of bone marrow cells to generate suppressor factors was confirmed in 1976 in experiments by A. Duve and S. Singhal. Mouse splenocytes ($10^7$) mixed with sheep erythrocytes and bone marrow cells ($10^5$-$10^7$) were cultivated on both sides of a millipore membrane. At high bone marrow cell concentrations, the immune response in the spleen cell population was markedly suppressed. The same effect was observed in experiments with separation of cells with a dialysis membrane. All this points to the small molecular sizes of the suppressor mediator.

While the stimulating effect of bone marrow cells on antibody synthesis is realized at the peak of the immune response, their suppressor influence manifests itself at early stages of this process, during its induction, i.e., within the first 24 hours of antigenic stimulation.

However, there is no unanimity among researchers as to the nature of cells responsible for the production of suppressor mediators. One possible reason for such discrepancies is that different types of bone marrow cells synthesize different suppressor substances. In our experience, the suppressor factor was produced by immature

B-lymphocytes. According to the data of A. Duve and S. Singhal, the suppressor effect of bone marrow cells on antibody formation is coupled with the activity of the so-called zero lymphocytes which do not carry on their surface specific markers for T- or B-cells. There is some evidence that bone marrow suppression is effected by immature cells of a myeloid type.

Although the true nature of bone marrow suppressors is still open to question, and many of their properties are currently obscure, the very fact of their existence leaves no doubt.

## Bone Marrow Is a Regulator of Immune Reactions

The discovery of stimulator and suppressor mediators produced by bone marrow cells enriched investigators with the new knowledge of the functional capabilities of this central organ of immunity. The existence of concrete molecular substances that communicate signals elicited by the bone marrow points to the ability of this organ to stimulate or suppress the antibody synthesis, to activate reserve cells or, if necessary, to restrict the proliferation of antibody-producing cell clones. The fact that the "orders" issued by the bone marrow are executed by its molecular messengers at different steps of the immune reaction provides compelling evidence of the involvement of this organ in various immunoregulatory processes.

The stimulator and suppressor effects of the bone marrow on the immune responsiveness of an organism differ in a number of features. As we know, the suppressor mediator displays the maximal activity during the induction of antibody synthesis, while SAP stimulates this process at the peak of the productive phase of the immune response. These mediators are produced by different populations of bone marrow cells, and they differ essentially in terms of their physico-chemical properties and mechanism of action. Studies with DNA synthesis inhibitors demonstrated that SAP production and its stimulating effect on antibody genesis are realized under conditions of blocked DNA synthesis, i.e., in the absence of cell division. Conversely, the suppressor mediator is synthesized by rapidly multiplying bone marrow cells, and its inhibiting action is directed against the proliferating precursors of antibody-forming cells.

It is well known that the vast majority of biological processes is controlled by antagonistic reactions, whose interrelationships proved for the equilibrium of a particular process. The immune response of an organism to the entry of a foreign particle (antigen) is one of such reactions. The regulatory cells involved in cooperative interactions which make the basis of any immune response, stimulate or suppress this process with the help of their molecular messengers, or mediators. Among these, the stimulator and suppressor mediators

of the T-system of immunity are by far the most extensively studied and best understood. T-lymphocytes were the first to be granted the "title" of regulatory cells.

The discovery of stimulator and suppressor mediators produced by the bone marrow has considerably extended our knowledge of the cells involved in the regulation of immunogenesis. Apart from stimulating or suppressing various immune reactions occurring in the organism in response to antigenic stimulation, the bone marrow seems to be involved in the functioning of more general immunoregulatory mechanisms. Thus, it is not unlikely that the suppressor factor "prohibits" the antibody formation on the territory of the bone marrow: the latter contains all cells necessary for antibody genesis to take place, i.e., T- and B-lymphocytes, macrophages, etc. An antigen enters the bone marrow. However, in case of antigenic stimulation, the process of antibody synthesis is intense only in peripheral lymphoid organs (i.e., spleen and lymph nodes). In the bone marrow, the antibody genesis is far less pronounced and takes place only at the ultimate steps of the immune reaction.

The role of stimulator bone marrow cells in immunogenesis is not confined to the enhancement of antibody synthesis at the peak of the immune response. The fact that SAP is produced in the course of normal metabolism of bone marrow cells and is always present in the organism points to other potentialities of this remarkable compound which require thorough investigation. Further developments in this field as well as a search for other factors generated by the bone marrow seem to be the most promising approaches to the problem of immunoregulation.

## Endorphines Are Molecular Messengers of the Nervous System

Regulatory and effector signals elicited by concrete molecular substances are vitally important for the normal functioning of various systems, tissues, and cells of the body. Recently, a new group of mediators—neuropeptides—has been identified. These molecular substances are generated by nerve cells; their main function is to communicate signals from the central nervous system to different cells and tissues of the organism.

One of such compounds, morphine, the main alkaloid of opium, is widely known as a hypnotic to relieve pain both in animals and humans. Strong analgesic effects may also be exerted by a great variety of other substances. Morphine-like compounds are synthesized by nerve cells, and they anaesthetize many physiological processes occurring in the organisms of healthy individuals. In contrast with morphine which is exogenous in nature, these substances got the name of endogenous opioids, or endorphines, i.e., opioids originating

within the organism. To brain opioid peptides, we may relate encephalines, i.e., endorphine-like substances of low molecular weight. Endorphines and encephalines are structural fragments of β-lipotropin, a polypeptide produced by the hypothalamus and the pituitary gland.

The effects of neuropeptides exceed manifold those of exogenous opioids. Thus, the analgesic effects of encephalines are hundreds of times as strong as those of morphine taken in the same dose. Another neuropeptide in this series is the so-called δ-peptide of sleep, whose molecular size is somewhat larger than that of endorphine; its molecule consists of nine amino acids. The δ-peptide in a dose as low as $10^{-6}$ g per kg of body weight induces deep sleep. There are neuropeptides that stimulate learning, accelerate memorizing, etc.

Neuropeptides, the mediators of the nervous system, interact with various cells by binding to their surface receptors. Specific receptors for opiates were detected in the nervous tissue in early 1970's. In subsequent years, such surface receptors were identified on renal cells, adrenal and pancreatic secretory cells, as well as on peripheral blood lymphocytes. These data suggest that neuropeptides which communicate signals from the central nervous system to different organs and tissues of the body function as neuromodulators. The identification and characterization of endorphines stimulated chemists and pharmacologists to unite their efforts in a search for synthetic analogs of these remarkable substances that would exert unalloyed stable analgesic effects. This requirement was dictated by economic reasons, since the isolation of natural endorphines and encephalines is a very complicated and expensive procedure: these substances are localized exclusively in brain tissues where their content does not exceed several micrograms. The deciphering of the structural peculiarities of individual neuropeptides has provided a way to the large-scale production of their synthetic analogs. In some of their most essential pharmacodynamic and pharmacokinetic properties, synthetic endorphines have many salient advantages over morphine.

Many aspects of the physiological activity of endorphines (e.g., mechanism of action, origin, biosynthesis, etc.) are still poorly understood. Thus, until very recently, there was an opinion that endorphines are synthesized in the pituitary gland by enzymatic cleavage of the β-lipotropin precursor molecule. However, the enzymes splitting β-lipotropin were also detected in large amounts in the smooth musculature of small intestine as well as in some endocrine glands. A few endorphine-like substances were identified in the blood. In the blood of hypophysectomized animals, β-endorphine is absent; its content in brain tissues, however, is unaffected by the surgery, which points to the different origin of these endorphines. In the blood and urine of humans, low molecular weight opiate-like

substances are present in a non-active state, e.g., within a complex with proteins.

Many problems relevant to the origin and functional significance of endorphines require further elucidation and experimental verification. A search for new sources of these indispensable compounds may culminate in discoveries of great theoretical and practical importance.

## Endorphine-Like Nature of SAP

In the sections above, we stressed the ability of SAP to stimulate antibody genesis at the peak of the immune response. Is this the main and single capacity of this mediator? Why, then, is it synthesized by bone marrow cells of non-immunized animals in the absence of antigenic or mitogenic stimulation? Can SAP, besides its immunoregulatory role, perform any other biologically important functions that are necessary for the normal life-cycle of an individual?

In collaboration with staff members of the Research Institute for Reflexotherapy and All-Union Center for Mental Health of the USSR Academy of Medical Sciences, we conducted a series of experiments aimed at determining the opiate-like activity of SAP, with two basic goals in mind: (1) to test the feasibility of functional similarity of bone marrow peptides to neuropeptides, and (2) to investigate and, perhaps, try to uncover some new properties of these compounds. One of the few experimental systems used in this study was a neurophysiological model.

In experiments with electric stimulation of cats, the pain stimuli were applied in the region of the dentinal pulp (algesic nociceptive stimulation) and the lower lip (non-algesic tactile stimulation); the electric potentials generated in response to stimulation in different zones of the large hemispheres of the cerebral cortex were recorded. The animals were then intravenously injected with SAP (10 mg per kg of body weight), and the whole procedure was repeated.

It appeared that SAP diminished the pain response, predominantly in the cerebral cortex area that controls emotional-behavioural pain reactions. Conversely, the non-algesic tactile algesthesia increased after SAP injections. The analgesic effect was maximal on the 40-60th min of stimulation and was eliminated by the 90th min after SAP injection.

Such selective suppression of nociceptive pain signals is induced by hypnotic morphine-like analgetics as well as by endogenous opiate-like substances—endorphines. Further evidence for the analgesic, endorphine-like nature of SAP was obtained in behavioural studies during the induction of the so-called "tail jerk" response in rats. Pain responses were evoked by stimulation with focussed light

("light pencil"); the onset of the "tail jerk" response was carefully recorded. Intraperitoneal injections of SAP (20 mg per kg of body weight) significantly increased the latent phase of the pain response. The analgesic effect was maximal on the 40th min after SAP injection.

An interesting peculiarity of these experiments was that SAP had absolutely no effect on behavioural responses of experimental animals. The dynamics of orientative reflexes through time, the motor activity, and the number of washings which characterize the emotional behaviour of animals, did not change after SAP injections. This finding points to differences in the mechanism of analgesic action of SAP and morphine: the former, in contrast with the latter, exerts an appreciable anaesthetic effect without having any conspicuous influence on the behavioural responses. The endorphine-like nature of SAP was also revealed in experiments *in vitro*, when the ability of SAP to bind to opiate receptors was studied.

$^{3}$H-labelled morphine (or metencephaline) and some time later, unlabelled SAP were added to brain cortex membranes placed into a test-tube. The ability of SAP to displace opiates from the sites of their binding to brain receptors was followed by the accumulation of the free unbound label. In this experiment, SAP appeared to compete with opiates for specific receptors. The displaced label content was twice as low, when bacitracin, an inhibitor of protease synthesis, was added to the reaction mixture. (Proteases are protein-splitting enzymes.) This implies that the ability of SAP to displace receptor-bound opiates is due to the peptide nature of the reaction components. Similar results were obtained in experiments with competitive displacement by SAP of $^{3}$H-metencephaline bound to the receptors of human peripheral blood lymphocytes.

Thus, direct evidence of the endorphine-like nature of SAP was obtained both *in vitro* and *in vivo*. This discovery offers entirely new approaches to the problem of immunomediators. Until very recently, little was known about lymphokins and monokins whose properties are very similar to those of SAP. There were only some vague indications of the similarity of the physico-chemical properties of thymosin and some brain polypeptides. Thymosin was shown to be able to bind to nerve cells; $\alpha$-thymosin was detected in brain tissue homogenates.

Not long ago, it was shown that interpherone isolated from human peripheral blood leucocytes can specifically bind *in vitro* to opiate receptors of the brain. When injected intraperitoneally to experimental animals, this protein exerts appreciable analgesic effects.

Taken together, these data provide convincing proofs of the endorphine-like nature of some immunologic mediators.

## Myelopeptides: Are They Neuroimmunomediators?

We have seen, in preceding sections, that the bone marrow, one of the key organs of immunity, is a treasure-trove of many mysteries.

This organ "gives birth" to all types of blood cells that have a common precursor—a haemopoietic stem cell. In mammals, the bone marrow is also a source of B-lymphocytes, the main participants of humoral immune responses terminating in antibody synthesis. Recently, the bone marrow has been shown to control immune reactions with the help of its molecular messengers—stimulator and suppressor mediators. And, finally, besides immunoregulatory factors, this organ can generate endorphine-like substances.

Studies on biological and physico-chemical properties of soluble factors produced in the bone marrow revealed the great functional heterogeneity of bone marrow mediators. As mentioned earlier, the endorphine-like activity was detected in the SAP-containing fraction. This fraction obtained by gel filtration of bone marrow cell supernatants on Sephadex columns appeared to be heterogeneous and during polyacrylamide gel electrophoresis and isoelectrofocusing on ampholines was resolved into six individual peptide subfractions.

Whether the endorphine activity is inherent in the whole SAP molecule or in its constituent peptides, is still unclear. Clues to this question may come from further analyses. But even now the enormous impact of bone marrow peptides on the immune and nervous systems is beyond doubt. We termed these substances as "myelopeptides" as opposed to neuropeptides that are synthesized by cells of the nervous tissue. Developments in this field will undoubtedly lead to the discovery of other myelopeptides as well as to the elucidation of their structural and functional roles.

It seems likely that neuro- and myelopeptides intermediate various relationships between the immune and nervous systems. There is evidence that endorphines, encephalines, and exogenous opiates can bind to lymphoid cell receptors. Morphine decreases the content of T-lymphocytes in peripheral blood, while metencephaline exerts an opposite effect. Opiates change the level of cyclic nucleotides and thus alter the degree of their activation. Within an animal organism, opiates sharply diminish the absolute content of T- and zero-lymphocytes in peripheral blood, leaving B-lymphocytes unaffected. The results presented herein testify to the ability of neuropeptides to control the course of the immune response at the expense of their binding to specific receptors situated on the lymphoid cell surface.

It is not unnatural to suppose, therefore, that there exists also a feed-back transmission of signals from the immune to the nervous system. How does such signal system work? Answers to this question may only come from the analysis of concrete mediator substances

involved in this process. In our opinion, myelopeptides that possess the endorphine-like activity and reveal, in addition, immunoregulatory properties have every reason to be regarded as possible candidates for mediators whose major function is to convey information from the immune system to nerve cells. If that were true, the problems of neuroimmune regulation could be further analyzed at the level of concrete molecular substances mediating the relationships between the two systems. It remains to be hoped that future studies will lead to the discovery of many more of the unique capabilities of myelopeptides and soon these wondrous substances will become salubrious to man like neuropeptides.

Today, the problem of immunomediators has become one of the most favoured fields of research. Alongside a search for new humoral factors, studies are currently underway to determine the chemical and biological activity of the already known mediators (also on a comparative basis), to elucidate various relationships between the mechanisms of their synthesis and the genetic system of the cell, etc. Further developments in this field will be directed at the investigation of their mechanisms of action and the refinement of the existing techniques of their isolation in a highly purified state.

Significant progress has been achieved in the last decade in the field of cell hybridization. The principle of this method consists in the fusion of a mediator-secreting cell with a reinoculated tumour-associated lymphoid cell. The resulting hybrid thus acquires two valuable properties: it can produce a single type of a mediator and is capable of uncontrolled proliferation. The use of this technology has provided a new approach to the production of monoclonal cell lines capable of secreting a single mediator type. The possibility of obtaining excessive amounts of pure mediator substances is of tremendous importance—both theoretical and practical—for applications in clinical medicine for immunocorrections and immunotherapy.

# Vaccines of Tomorrow

## The Pasteur Method of Development of Vaccines

Over a century ago the great Louis Pasteur proposed the basic principle of vaccination. It consists in preventive inoculation that ensures immunity against infectious diseases. Attenuated or killed microbes are administered to the organism of man or animal. The organism easily resists their action. The blood develops immune proteins, called antibodies. These are capable of destroying not only attenuated but also full-strength infecting agents by neutralizing their toxins. Pasteur attenuated the virus of hydrophobia in man thus preparing a vaccine against this disease. He attenuated the bacillus of anthrax and saved humanity from this awful infection. It is by using the Pasteur method that vaccines were prepared to counter plague, poliomyelitis, tick-borne encephalitis, measles, and a number of other infectious diseases. Smallpox that until recently took lives of millions of people is now eradicated all over the world.

The vaccination principle formulated by Pasteur lives on, immensely benefiting mankind. More and more infectious diseases are being stamped out. Fewer and fewer are diseases that remain unconquered. Yet a number of outstanding problems do exist.

**Live vaccines.** As early as ancient times it was known that people who had had infectious diseases became immune to them. For instance, a man who had had smallpox would not be infected by it again. Experience gained during the centuries was utilized. In various countries experiments were conducted to administer material from smallpox-stricken cases to healthy individuals. This material, dried and crushed crust from lesions, was the first live vaccines to be used from purely empirical experience. However, such methods of protective inoculation failed. As a rule, the inoculation brought about a grave, often fatal disease. Moreover, individuals who received smallpox material, became a source of infection.

Credit for the discovery of the first safe live vaccine goes to the English doctor Edward Jenner. A peasant woman had become ill with cowpox, a few characteristic vesicles forming on her hands. Matter from one of these was injected to an eight-year-old boy. Two months later the boy was inoculated for smallpox. The disease, however, did not follow. The inoculation had rendered the boy immune to one of the most fearful infections, smallpox. This kind of inoculation was termed vaccination (from Latin "vacca", a cow).

The term became generally accepted. Since then any prophylactic inoculation of an infecting agent has been so named. In 1798 Jenner published his "Inquiry into the Cause and Effects of the Variolae Vaccinae". This date can be regarded as the advent of the era of prophylactic vaccination.

Thus, Jenner taught humanity to be unafraid of smallpox. Yet neither he nor medical science of the time were able to offer a general method of prevention against any other infectious disease. No scientific approach, no theory existed. Science had to mature, humanity had to learn some more. Finally, Louis Pasteur had to be born in order to found, 85 years after Jenner's discovery, what is now known as immunology and to provide mankind with the principle of development of vaccines against numerous infections. 1881 was the year when the immunology was born. In May, 1881 Pasteur conducted an experiment showing that it was tenable to artificially attenuate the contagious effect of pathogenic microbes and to use attenuated infecting agents for protective inoculations. Pasteur prepared vaccines to counter chicken cholera, anthrax, hydrophobia and rabies. These all represented live attenuated cultures of the agents that induced the aforementioned diseases.

In order to obtain attenuated strains, prolonged growth is necessary of disease-producing microorganisms in the bodies of animals resistant to them, in tissue cultures or in vitro conditions, so as to change the genetic properties of the infecting agents. Such microorganisms cannot induce disease in the recipient. Yet they exhibit the same antigenic properties as the infecting agent and can reproduce in the vaccinated host organism. The net result is that the vaccinated person develops no clinical symptoms of the disease. Rather, specific immunity is induced against the disease caused by contagious microorganisms of the species from which the vaccine has been prepared.

**Dead corpuscular vaccines.** Dead vaccines are obtained by activating pathogenic bacteria and viruses by chemical or physical means. To obtain dead corpuscular vaccines requires no special growth of vaccine strains, unlike the preparation of live attenuated vaccines. It suffices to kill any most pathogenic type of infecting agents preserving their antigenic composition. Regretfully, dead vaccines representing intact bacteria or viruses often fail to elicit an effective immunity in contrast to live attenuated vaccines.

**Anatoxins.** These are bacterial toxins that have lost their toxic activity owing to the special treatment. In so doing, however, they have preserved their antigenic and immunization properties. The common procedure is to inactivate the bacterial toxins with formalin or by thermal treatment. Apart from antigens needed for immunization, anatoxin preparations include a large proportion of ballast substances which cause allergies and other harmful side effects. Wide

use is currently made of anatoxins of agents of gangrene, diphtheria, tetanus, and some other diseases.

**Subunit vaccines.** It is not uncommon that live attenuated or dead vaccines, apart from a protective action, demonstrate strong side effects. They may produce, e.g., a general toxic effect on the organism, give rise to allergies etc. Immunization with viral vaccines inevitably entails introduction into the organism of huge quantities of alien genetic material (nucleic acids) as well as antigens of embryonic tissues on which viruses are grown. This is why investigations have been started in recent years to isolate (in particular, from viruses) specific antigenic fragments which produce the vaccinating effect yet show no general toxic properties. Such "purified" antigens became known as subunit vaccines. For example, a subunit anti-influenza vaccine represents either a protein (glycoproteid) hemagglutinin, a neuraminidase or a mixture of these glycoproteids. These molecules are located at the surface of viral particles and are their principal antigens responsible for the immunization effect.

Investigations are currently in progress with the intent to obtain subunit vaccines to counter influenza, viral hepatitis and other infectious diseases. It will be noted at this point that subunit vaccines are typically weak antigens and thus induce indeterminate immunity. For this reason the developers of subunit vaccines are faced with a most urgent task to find pathways to convert these vaccines to effective immunogens.

**So-called "chemical vaccines".** This type of vaccines is wrongly described as chemical. In fact, they are not those definite chemical compounds whose structure and composition are known. What is termed "chemical vaccines" is, as a rule, a mixture of various components isolated from microorganisms. They exhibit an ability for the vaccinating action. "Chemical" vaccine preparations are either endotoxins (bacterial toxins freed when bacteria are broken down) or they may be metabolic products of microbes cultivated in special nutrient media. The use is being made of typhoid and paratyphoid vaccines. Regretfully, even the most purified vaccinating preparations of this type contain a multitude of ballast substances. These lead to formation of useless antibodies, including ones that induce allergies and other harmful side effects.

**Unconquered infections (limitations of the Pasteur method).** Thus, the scientific method proposed by Louis Pasteur in 1881 still lives on. In order to induce immunity it is necessary to attenuate the microbe, kill it or isolate from it the most responsible antigens and use these for immunization.

It might seem that the best vaccine would be a preparation made up of the most active microbial antigens. Yet, for some unknown reason, the antigens isolated from a microbe and purified do not in many cases work as efficiently as do live attenuated microbes. What

is more, it is unfeasible to prepare vaccines to combat some diseases even from live attenuated microbes.

Man's immune system, as it were, is unable to provide resistance to a number of antigens and microorganisms. No effective vaccines have as yet been prepared against influenza, dysentery, malaria, venereal diseases (gonorrhea, syphilis), and others. An effective anticancer vaccine is seemingly a dream of the distant future. The principle formulated by Pasteur does not work in this case. How is immunity against such diseases to be stimulated? Are new approaches needed? Are researchers looking for them? Well, science is always in search for more effective and promising pathways. What new principles must be relied upon to develop a vaccine to combat such an infection that evokes no immunity even after a genuine disease, to say nothing of the administration of an attenuated contagious agent? A person who has had smallpox or measles or has been vaccinated against these diseases will never have them again. In contrast, one may have influenza yearly—no immunity is induced. The most popular, generally accepted explanation is that influenza virus constantly changes, varying its proteins, i.e., antigens. A man will develop immunity against one strain of the virus, yet a year later another strain may appear. The explanation is convincing but not the only one. Rem Petrov has put forward a hypothesis that most people belong to so-called low-responder genotypes with respect to influenza and other "unyielding" infections. What does this mean?

Some 15 years ago immunologists discovered previously unknown genes, so-called immune response genes (Ir-genes). As is known, all characters of an organism are determined by the activity of some genes or others. The immune response genes control the power of the immune reaction, i.e., they are responsible for the production of antibodies against a certain antigen commonly represented by a protein of the viral or microbial envelope. There is a great number of immune response genes. An organism may vigorously react against one antigen and feebly against another. In contrast, another organism may exhibit a reverse response. If for some unknown evolutionary reasons most humans belong to low-responder genotypes with respect to influenza-inducing agents, it is untenable to develop a vaccine along conventional lines. One must learn to convert a genetically low-responder organism to a high-responder one. One should develop new types of vaccines, whose action would be independent of immune response genes. Such vaccines should produce in any organism the immunity to any infection.

### Artificial Antigens

Artificial antigens have played an important role in revealing the principles of the activity of the immune system. Moreover, they provided evidence as to genetic control over the intensity of

the immune response. Most immunologists, microbiologists, organic chemists, and other workers associate the term "artificial antigens" with two principal categories of macromolecules.

The first category includes high-molecular weight compounds, say, proteins or polysaccharides modified by certain chemical groups. An example can be provided by Landsteiner's proteins to which some haptens are attached by means of chemical bonds. Viewed in this context, any hapten-protein, hapten-polypeptide or hapten-polysaccharide complex is an example of an artificial antigen with a given haptenic specificity. The second category of compounds includes man-made macromolecules that exactly follow the structure of the native antigen. For example, after decoding the structure of erythrocyte group antigens (A, B, Lewis, etc.) they were synthesized. In this sense synthetic insulin is an artificial antigen. Closely linked to this category of artificial antigens are synthetic macromolecules. These, even though they do not represent exact replicas of definite nature antigens, are their analogs. Synthetic polypeptides, linear or branched copolymers of amino acids, provide examples of such artificial antigens. These synthetic macromolecules reproduce the structure of natural macromolecules. Credit goes to the work of M. Sela and B. Benacerraf. They have immensely contributed to the development and study of such antigens.

A third, new type of artificial antigens has, as important functional fragments, chains of polyelectrolytes whose analogs do not naturally occur. They were first synthesized and studied by Soviet chemists and immunologists led by the present authors, Kabanov, Petrov, and Khaitov. We will dwell at some length on these compounds.

The fifty years of utilization of man-made antigens for experimental immunology have provided theory with most important findings. By means of Landsteiner's antigens the molecular basis of antigenicity has been determined. The essential role of determinant groups in this phenomenon has been shown. Moreover, the specificity of active centres of antibodies has been established as well as their size, the character of antigen-antibody bonds, etc. Investigations using synthetic polypeptides and other analogs of natural antigens made it possible to gain an insight into genetic control over antibody genesis and delayed-type hypersensitivity. They also permitted the discovery of immune response genes, Ir-genes. Through the use of artificial antigens that do not naturally occur and are developed on the basis of synthetic polyelectrolytes it became possible to develop promising methods of phenotypic correction. These allow conversion of individuals with a low level of immune response to ones that manifest a high level of response. All these achievements laid the foundations for the creation of vaccines of the future and promoted a significant break-through in the problem.

Pasteur proposed the principle of the preparation of vaccines. His main method of immunization was to employ attenuated strains of contagious microorganisms as immunogens. The development of this method, its modifications and improvement can be summed up as follows.

The essential novelty of today's state of the art lies not only in the fact that we are witnessing the dawn of artificial macromolecules that exhibit necessary and only necessary antigenic determinants. In fact, it is nothing but an extension of Pasteur's principle. The thing is that if an organism, because of its genetic features, is unable to unambiguously exhibit an immune response to a particular agent, antigen or antigenic determinant, neither will its synthesized analog stimulate immunity. What is important at present is to find the means of a phenotypic correction to ensure the immune response even in individuals insensitive to the given antigen. The principal method of achieving this is to construct such artificial macromolecular complexes that should include both the required antigenic determinants and an activating (adjuvant) structure. The latter, not being immunogenic, should, however, ensure a phenotypic correction.

Dealing with immunology and immunogenetics of synthetic immunogens, we endeavoured to shed as much light as possible on the advance in the development, study and use of antigens of this type. In addition, we tried to outline eventual applications of the established mechanisms to prepare vaccines of the future.

**Synthesis of natural antigens.** Most naturally occurring antigens (proteins, glycopeptides, polysaccharides, etc.) are complex molecules eliciting a wide-range immune response. The resultant antibodies are directed against each separate antigenic determinant included in the structure of the complex molecule. Even immunization with a uniform and chemically pure protein antigen is likely to generate several types of antibody, each of them being complementary to one of the determinants of this antigen. Therefore, when speaking of synthesis of some antigens, one implies that artificially created is one of the determinants of a natural molecule rather than its entire structure.

*Antigens of blood groups.* Hematology witnessed many cases when blood transfusion from one individual to another regardless the blood groups of the donor and the recipient led to a dangerous immunological reaction. This sometimes caused a lethal result. The fact is that most people's blood contains antibodies capable to agglutinate and destroy the red blood cells (erythrocytes) of many (but not all) other individuals. In 1900 Karl Landsteiner separated the erythrocytes into four groups: A, B, AB, and 0. The group specificity of erythrocytes, it turned out, is determined by special antigens, A and B, inherited according to the Mendelian laws. The erythrocytes

| *Type of Immunogen* | *Method of Preparation* |
|---|---|
| I. Vaccines and anatoxins | |
| Live attenuated vaccines | Attenuation of virulent activity as a result of prolonged growth of infecting agent outside organism of animal susceptible to the given infection; selection of spontaneous or artificial mutants; recombination. |
| Dead corpuscular vaccines | Inactivation (destruction) by chemical or physical means of pathogenic bacteria and viruses. |
| "Chemical" vaccines | Isolation of immunogenic components from microorganisms (toxins and other antigens). |
| Anatoxins | Inactivation as a result of special treatment (formalin and other means) of toxin activity preserving antigenic and immunization properties. |
| Polyvalent (associated) vaccines | Preparation of a mixture of immunization components of several agents or different serologic types of one agent. |
| Adsorbed (deposited) vaccines | Development of preparations of "chemical" vaccines and anatoxins deposited on mineral or other components (aluminium hydroxide, polysaccharides) exhibiting deposition and adjuvant properties. |
| II. Artificial immunization molecules synthesized according to the principle of naturally occurring analogs | |
| Conjugated (hapten-carrier) antigens | Chemical compound of non-immunogenic (haptenic) or weakly immunogenic determinants with soluble protein and polysaccharide carriers as well as with insoluble high-molecular carriers. |
| Synthetic polyamino acid antigens | Artificial synthesis of polypeptide chains — linear homopolymers or copolymers with branched chains. |
| Conjugates of synthetic polyamino acid antigens with synthetic adjuvants | Chemical compound of synthetic polypeptides with synthetic bacterial glycopeptides of muramyldipeptide type. |
| Synthetic polysaccharides | Artificial synthesis of polysaccharide determinants of microbial and tissue antigens. |
| III. Artificial immunization molecules without natural analogs | |
| Complexes and conjugates of haptens and antigenic determinants with synthetic unnatural macromolecules actively modulating immune response | Synthesis of complex macromolecules made up of necessary antigenic determinants and a polymer ensuring adjuvanticity, T-independence and Ir-independence of the entire macromolecular complex. |

species of the bacteria group. These are tyvelose (in *S. typhi*), paratose (in *S. paratyphi*), colitose (in *E. coli*), etc. The structure of the specific part of a 0-antigen is shown in Fig. 34.

N. K. Kochetkov and his co-workers of the N. D. Zelinski Institute of Organic Chemistry (the USSR Academy of Sciences) have succeeded in finding the procedures for chemical synthesis of specific bacterial polysaccharides built up of recurring olygosaccharide links. Such synthesis makes it possible to obtain an appreciable amount of haptens in a form convenient for attachment to a carrier. The authors have first used high-molecular weight proteins as carriers. These proteins, after administration of their conjugates with synthetic

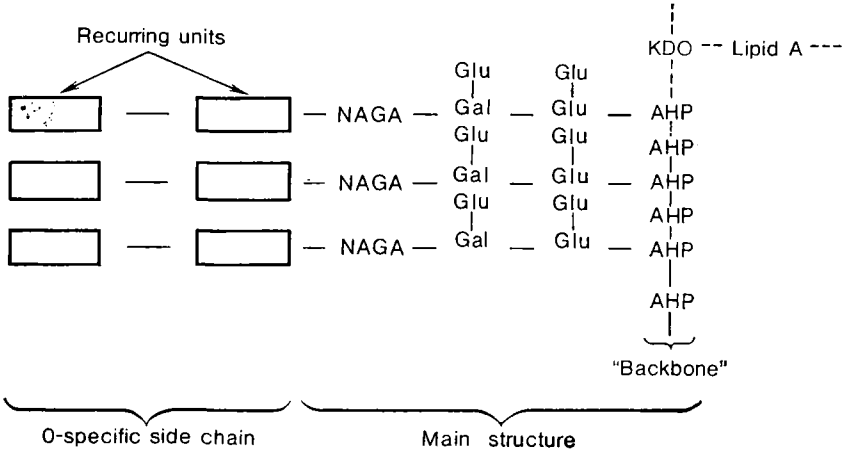
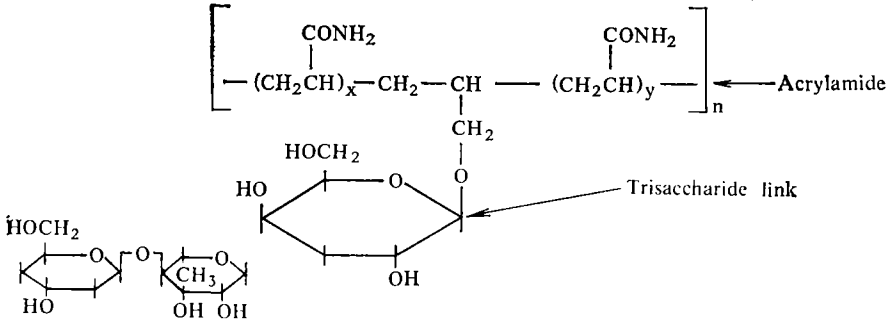



**Fig. 34.** Structure of specific part of 0-antigen of intestinal typhoid group.
KDO—ketodesoxyoctionic acid; AHP—aldoheptophosphate; Glu—glucose; Gal—galactose; NAGA—N-acetylglucosamine

olygosaccharides in a complete Freund's adjuvant (followed by a repeated administration without the adjuvant) evoke rather a high degree of immune response. Moreover, the experimentators made another move forward. They made a creative attempt to diverge from the conventional method of linking olygosaccharide fragments to immunogenic protein carriers.

In 1982 N. K. Kochetkov and V. I. Pokrovski with coworkers synthesized and tested for immunity a hydrocarbon containing an artificial antigen with a specificity factor 0; 3 of the *Salmonella* serological group E. They did it by transferring the hydrocarbon determinant to a high-molecular state following copolymerization with acrylamide. This type of antigen contains as a determinant a complete trisaccharide recurring link of the bacterial (*Salmonella newington*) polysaccharide. In addition, it specifically interacts with

antiserum of rabbit immunized with the corresponding *Salmonella* group (anti-0; 3; 10 serum from rabbit immunized with *S. anatum* and anti-0, 3, 15 serum from rabbit immunized with *S. newington*). This suggests the presence of a monofactor specificity 0; 3 in the copolymer.




The Swedish researchers S. B. Svenson and A. A. Lindberg have synthesized a number of 0-antigen-specific saccharide-protein conjugates which are, in fact, artificial salmonellous vaccines. Such vaccines save mice administered with a lethal dose of *S. typhimurium*. In these studies synthetic saccharides were conjugated with native proteins, in particular, bovine serum albumin (BSA) thus giving rise to T-dependent antigens. In this case, the T-dependence is a disadvantage of such artificial vaccines. In what follows we will dwell on the problem of genetic control over the immune response. At this point we will mark in anticipation that the power of the immune response is strictly controlled genetically. An individual, bearing a gene of low immune response to a particular antigen is unable to produce antibodies against this antigen. At the same time the immune response genes express their activity at the level of T-lymphocytes. Therefore, it is not by chance that, on converting a T-independent polysaccharide antigen to a T-dependent one by conjugating it with BSA, the Swedish investigators noticed a protective effect only in such mouse strains that are genetically high responders to BSA. The injection of such T-dependent artificial vaccine to low-responder recipients, e.g., C57BL mice, induces no immunoeffect whatsoever.

*Lysozyme and its subunits.* A perfect example of antigen engineering can be provided by molecular immunologic experiments conducted by M. Sela. Their objective was to synthesize individual peptide fragments of lysozyme likely to be antigens. The experiments were to prove that antibodies are produced to act not against the entire molecule, say, of a viral or microbial protein, but, rather, against some particular determinants of the macromolecule, its definite unique structures.

It will be recalled that a hen's egg white contains lysozyme, an antigen convenient for investigations. Its primary spatial structure is well studied. M. Sela has isolated a fragment of a lysozyme molecule. It is made up of residues of 24 amino acids successively linked to one another to form a so-called "loop". Such an isolated loop

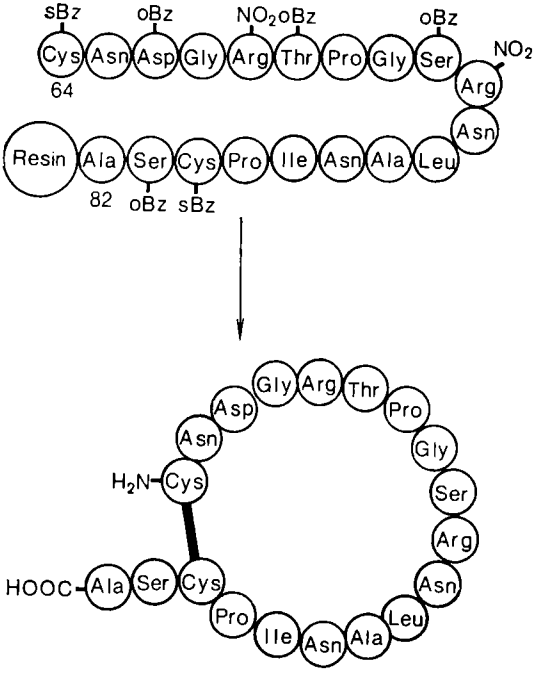



**Fig. 35.** Synthesized peptide "loop" of lysozyme containing amino acid residues 64-82.

In circles are amino acid residues. Above is broken loop, below is loop broken along line Cys-Cys. This lysozyme fragment is responsible for antibody production in organism

attached to a synthetic carrier macromolecule and introduced into an organism evokes the generation of antibodies against lysozyme. As a carrier, artificial analogs of a protein molecule, polyalanine and polylysine were used. Unlike native proteins that are chains of different amino acids, polyalanine and polylysine are chains all links of which are similar and made up of solely alanine or lysine amino acid, respectively. Now comes the most interesting thing. Since the amino acid sequence of the "loop" had been known, several of its separate components were synthesized. Immunization with these separate synthetic products attached to the carrier showed what

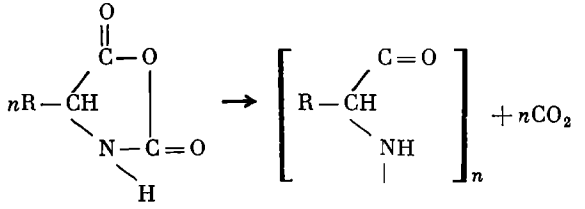
particular constituent was the most important and what fragment was responsible for the formation of antibodies (Fig. 35). At this, antibodies thus obtained did not differ from the ones produced in response to the native product.

M. Sela gave a convincing proof that it was tenable to utilize synthesized analogs of native antigens in order to obtain antibodies that would react to native antigens. Apparently, similar results can be obtained using a variety of proteins whose amino acid sequence is known. Advance in this field will be warranted by progress made in the study of the primary and tertiary structures of protein walls of different viruses and microbes.

**Synthetic polypeptide antigens.** Many protein antigens are made up entirely of amino acids. No wonder, therefore, that immunologic specificity of such antigens should be connected with certain amino acid groups. Polypeptide antigens synthesized by now are exact analogs of native antigens. They evoke the same immunologic reactions as do the native ones. Also they can be T-dependent and T-independent. At the same time, the use of synthetic polypeptide antigens for immunologic and immunogenetic investigations has a major advantage. It lies in the fact that such antigens exhibit a limited spectrum of determinants with individual specificity. Antigen models have made it possible to detect genes that exert control over the immune response specific for a particular determinant. The largest contribution to the creation of synthetic polypeptide antigens is due to M. Sela.

Basing on synthetic polyamino acids, employing them as a carrier, it is possible to obtain immunogens that are capable of producing antibodies endowed with practically any desired specificity. Immunogens are synthesized by attaching to polypeptides such substances as sugar, nucleotides, haptens, etc.

*Linear polypeptides.* Linear polyamino acid antigens are obtained by polymerization of amino acid N-carboxyanhydrides:



Investigations started in 1963 by American scientists relied on the development of synthetic polypeptides with a sufficiently great molecular mass to manifest antigenicity, if any. A study of the antigenic composition of polypeptides with the previously known number of amino acids involved has made it possible to reveal the sig-

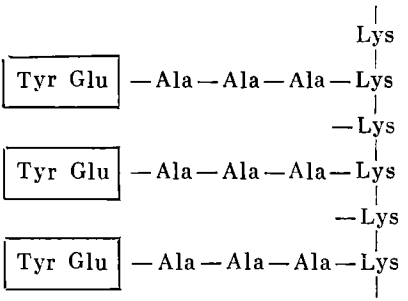
nificance of the primary structure of the protein molecule in order to determine antigenic specificity. P. Maurer and his co-workers have analyzed antigenic activities of various amino acid copolymers. These included polyamino acids made up of single amino acid; polypeptides made up of two amino acids in different combinations, e.g., glutamine—tyrosine (90 : 10) or glutamine—alanine (6 : 40) etc.; copolymers made up of three amino acids, e.g. glutamine—lysine—alanine (42 : 28 : 30 or 57 : 38 : 5), glutamine—lysine—tyrosine (58 : 38 : 4), etc. Linear homopolymers did not appear to exhibit antigenic activities. Clearly, a multipeptide chain made up of the same multiply recurring amino acid is unlikely to manifest any alien elements, no specific pattern of molecule forming. Dipolypeptides and notably tripolypeptides possess antigenic activities. At this, the antigenicity of different polypeptides varies. Glutamine—lysine—alanine in the ratio 42 : 28 : 30 is much more antigenic than in the ratio 57 : 38 : 5. It seems that different ratios of the three components give rise to a different number of possible sequence combinations that determine alien elements.

All the polymers obtained were subjected to cross reactions in order to estimate their antigenic specificity. It turned out that all the polymers showed both similarity and antigenic difference. The addition to a polypeptide of amino acids containing aromatic rings (tyrosine, phenylalanine) led to a new kind of specificity. The antigenic similarity of various copolymers stems from the presence of molecular segments with the same sequence of amino acids. The more amino acids copolymers contain, the greater is the number of wide-range antibodies. The molecular mass of polypeptides must be at least 3,500 daltons. The best results were yielded by a polymer with a molecular mass of 80,000 daltons.

*Branched polypeptides.* Significant progress has been made not only in gaining an insight into the structural basis of antigenicity but also into understanding of the genetic basis of the response to antigens. This became possible due to experimentation work with branched (multichain) copolymers, in which linear polypeptide chains are attached to a polyfunctional nucleus ("backbone").

It is possible to obtain synthetic branched polypeptides by polymerization of N-carboxyanhydrides of amino acids on synthetic polypeptides, e.g., on polylysine, polyornithine or copolymers containing these basic amino acids. Such artificial antigens that have as constituent elements 4 amino acids were prepared in 1965 by H. O. McDevitt and M. Sela. Polyalanine side chains were attached to a polylysine chain, tyrosine and glutaminic acid residues being

attached to their terminal group:




In the first case the antigen had a structure (T, G)-A-L, in the second (His, G)-A-L. When phenylalanine was used instead of tyrosine, a third antigen was obtained (Ph, G)-A-L. The carrying function is performed by a polylysine chain, the antigenic determinants are formed from tyrosine, glutamic acid or phenylalanine, glutamic acid. It is by means of these synthetic antigens that the primary gene of immune response in mice has been found (Ir-1-gene).

*Sela's views on tomorrow's vaccines.* Speaking at a conference held in Paris to commemorate the 150th anniversary of Louis Pasteur's birth, Michael Sela, a well-known immunochemist, delivered a report on the development of antiviral vaccines in the future. Even though over a hundred years passed, he said, after Pasteur first formulated the principal method of vaccine preparation, today's vaccines and vaccination procedures little differ from the ones proposed by the great Pasteur. Indeed, frequently occurring complications induced by vaccinations as well as the ineffectiveness of vaccines against some infectious diseases point to the urgent need in new approaches and novel vaccination principles. It does not mean that the Pasteur method has outlived itself. Yet ever more promising methods, new pathways are needed in order to develop vaccines of an essentially new type. Science is constantly looking for more efficient and promising approaches. Is there at present any hope to develop vaccination materials of a new type? It seems there is.

Progress attained in chemical synthesis of polyamino acid antigens has opened up new possibilities for obtaining artificial vaccines that contain artificial antigen determinants of a particular infecting agent. A most interesting and peculiar approach to solve this problem has been suggested by M. Sela. He proposed to synthesize unique molecular structures (determinants) of responsible (protective) antigens, each of such structures recognizing a particular infecting agent. According to M. Sela, it would be possible to construct a macromolecule from several such fragments. Macromolecules of this type would represent a synthetic analog of a polyvalent vaccine which ensures immunization against several pathogens at a time.

Is this feasible? Yes, it is. Currently, however, this is only possible for such antigens the immunization potency of which is not lowered with using killed vaccines or their constituents. For separate antigens the question remains open. In fact, M. Sela himself believes that, if in all cases of vaccination only attenuated viruses showed a universal effect and killed vaccines were typically ineffective, then the synthetic vaccines suggested by him would be of no interest. In contrast, should killed vaccines eventually become important for immunization, Sela's principle could be relied upon for preparation of effective synthetic vaccines.

What is the essence of Sela's approach?

The specific feature of the immune system is this. Vaccination against various infecting agents evokes the production in the organism of antibodies against specific fragments of the antigen to be found at the surface of the infecting agent. In other words, the organism produces antibodies to combat not the entire virus or microbe, but rather, certain unique molecular structures. Such structures (determinants) may be of a polysaccharide or polypeptide nature. In the estimation of antigenic specificity of these determinants the crucial factor is steric conformation (three-dimensional structure of the molecule).

Antibodies against native proteins manifest a sluggish reaction to the same proteins upon denaturation. On the contrary, antibodies against denaturated proteins do not usually crossreact with the same in a native state. It is attributable to the fact that a native protein's antigenic determinants primarily mirror the molecular structure. In Sela's view, the antiviral antibodies are frequently directed against the determinants of the viral protein coat. Probably, similarly to other native proteins, these possess conformation-dependent determinants governed by the native structure of the coat proteins. Viewed in the context of these findings, it was most essential to clear up how feasible it is to induce the synthesis of antibodies specifically directed against some unique limited region of the macromolecule and concurrently against the native protein. M. Sela and co-workers began their studies by staging elegant molecular immunologic experiments with the objective of synthesizing a fragment of lysozyme and the fragments described above.

An example of a synthetic "loop" of lysozyme demonstrates the possible use of the entire synthetic molecule to produce antibodies reacting with the native protein. At the same time these antibodies would be directed against the unique conformation-dependent limited region of the protein. Undoubtedly, similar results could be obtained for other proteins, whose amino acid sequence is known. This may occur, e.g., when "constructing" synthetic molecules that would evoke an immune response to definite viral coat proteins. Progress in this discipline will depend on advance to be attained in the study

of the primary and tertiary structures of protein coats of different viruses and microbes.

The first attempt to obtain a synthetic vaccine to combat a virus proved to be a success. A fragment was synthesized of the protein coat of MS-2 coliphage to destroy *E. coli*. In fact, this phage is a synthetic vaccine. It is RNA-containing virus whose capsid contains 180 identical subunits of protein coat with a molecular weight of 13,700 daltons each and a separate molecule of another protein defined as A. The protein coat contains a separate polypeptide chain of 129 amino acid residues successively linked up with one another. The coat protein of MS-2 was cleaved with cyanogen bromide to yield three fragments, possessing the sequences 1-88 ($P_1$), 89-108 ($P_2$), and 109-129 ($P_3$), respectively. The peptides $P_2$ and $P_3$ were antigenic. Therefore it is the peptides corresponding, in terms of amino acid sequence, to $P_2$ and $P_3$ that were synthesized. The synthetic $P_2$ and $P_3$ were attached to a polyamino acid carrier, polyalanine. The synthetic $P_3$ did not cause the production of antibodies with a phage-neutralizing activity. However, immunization of rabbits with the synthetic antigen $P_2$ under conditions of an additional stimulation by means of an adjuvant* stimulated the synthesis of antibodies with a virus-neutralizing activity. The resultant antibodies did not differ from the ones produced in response to the vaccination by the intact virus or by the antigen isolated from its coat. The $P_2$ chemically bound with a water-soluble adjuvant isolated from mycobacteria (muramyldipeptide) likewise exhibited a capacity to synthesize virus-neutralizing antibodies.

Thus, it was shown for the first time that a completely synthetic antigen may be used to evoke an immune response leading to viral neutralization provided the antigen is administered in the adjuvant.

Sela's laboratory has recently synthesized a peptide fragment (the sequencies 91-108 of hemagglutinin) of an influenza virus antigen. When injected to rabbits, this peptide attached to carrier protein elicits the formation of antibodies destroying the influenza virus. Attempts to protect mice infected with a lethal viral dose using the synthetic vaccine were successful as often as not.

The important advantage offered by Sela's approach is that the materials to be obtained would be free from ballast substances that contaminate all today's vaccines without exception. In fact, killed microbes (or proteins, polysaccharides and other compounds isolated from them) include hundreds of antigens. Modern vaccines typically contain as little as 1% of relevant antigens. This means that, on vaccination, the immune system would have to produce anti-

* Immunogenesis stimulant consisting of a mixture of oil, water and tuberculosis mycobacteria.

bodies against ballast antigens. Thus 99% of the immune system's output would go for nothing. It is these unnecessary antibodies that cause complications (allergies, etc.) upon vaccination. Another advantage of this synthetic approach is the possibility to design postulated vaccination molecules meeting the requirements. Say, 5-10 certain (determinant) characteristics of various agents are "attached" to one synthetic macromolecule and employed for immunization. All these determinants will have to replace contemporary vaccines with a very limited number of correspondingly designed macromolecules. It appears, thus, that methods of molecular engineering may be utilized to "construct" most diversified versions of polyvalent synthetic vaccines. It seems a dream today, as M. Sela put it. Will this dream come true?

It should be pointed out that the synthesis of individual molecular structures of responsible antigens and their attachment to one macromolecule are far from being the final step in the preparation of effective vaccines. M. Sela proposes to borrow from nature the patterns for the development of synthetic vaccines. In his report to the Third International Congress of Immunologists held in Sydney in 1977 he said that we must mimic nature not as a whole but in parts. It is suggested that natural molecular structures be copied using methods of molecular engineering. Yet it is not known if this principle will be successful for the preparation of synthetic vaccines to combat all microbes and viruses. If we recall that antigens isolated from microorganisms (and it is copies of these antigenic fragments that Sela proposes to create) fail to evoke as efficient immunity as do live attenuated microbes then this regularity would be likely to show in "artificially assembled" vaccines. Moreover, there are many diseases against which vaccines from live attenuated microorganisms cannot as yet be prepared. It is attributable also to the fact that an active part in antibody production is played by the genotype (immune response genes). Should a given individual possess a low response gene to a certain antigen, his immune system would be unable to produce antibodies, whatever vaccination may be.

Thus there emerges another problem most difficult to solve: artificial vaccines of the future must exhibit a capacity to stimulate the formation of antibodies against various antigens in any organism even in low-responder or non-responder genotypes.

## Genetic Control over the Immune Response

The problem of genetic control over immunity is as old as the world. As long ago as antiquity it was noticed that different individuals demonstrated different susceptibility to the same infecting agents. There exist breeds of cattle, pigs, poultry, rodents that are resistant or non-resistant to viral and bacterial infections.

It turned out that identical, uniovular, twins are more frequently liable to the same infections diseases that non-identical ones. However, only during the last two decades was a precise quantitative analysis conducted of genetic control over the immune response. This latter is the capacity of an organism to produce specific proteins (antibodies) directed against particular infecting agents. It has been proven that the capacity to respond (or to unrespond) to some particular antigen as well as the level of the immune response are genetically coded. To put it otherwise, the same organism may be "potent", showing a higher response in respect to one antigen and concurrently "feeble", with a lower response in respect to another antigen. The capacity to respond powerfully or sluggishly to the given antigen is inherited according to the genetic Mendelian laws. This property is under control of the immune response genes. What is the way they operate?

**Immune response genes and the scene of their action.** The level of the immune response to the administration of natural antigens usually carrying a variety of differently specific determinants is governed by several genes. For this reason a precise quantitative analysis of gene control over immunogenesis is not easy to conduct. It is because of this that genetics of the immune response has been studied using a model of immunization with synthetic polypeptide antigens, which cause, as already reported, a response of limited specificity.

In 1963 some work was done that involved an artificial antigen, a conjugate of DNP hapten with a synthetic polypeptide—poly-L-lysine (PLL). This study made it possible to identify a first specific immune response gene (PLL-gene). The DNP-PLL conjugate was employed to immunize guinea-pigs of two genetically pure strains, 2 and 13. It turned out that strain 2 guinea-pigs exhibit a pronounced immune response to immunization with DNP-PLL. In contradistinction, the same immunization had no effect whatsoever in the case of strain 13 guinea-pigs. These failed to generate antibodies against DNP-PLL. Further investigations went along purely genetic lines. First filial generation was obtained by crossing strain 2 animals to strain 13 ones. The next step was to obtain second generation and so-called backcross hybrids. It appeared that the capacity to respond powerfully or sluggishly is inherited in strict accordance with the genetic Mendelian laws and is under control of one self-sustained gene. A substitution of the DNP hapten by any other hapten in the PLL molecule led to no change in the immune response with responding and unresponding guinea-pigs. For this reason the gene was said to be a PLL-gene. Professor B. Benacerraf who led the work was awarded the 1980 Nobel prize for medicine and physiology for his discovery of the immune response genes.

The first immune response gene in mice was discovered in 1965

by H. O. McDevitt and M. Sela. They have shown that the capacity of mice to develop antibodies against a synthetic antigen (T, G)-A-L is controlled by a specific gene localized on chromosome 17. This gene was named an Ir-1-gene (immune response gene 1). A multitude

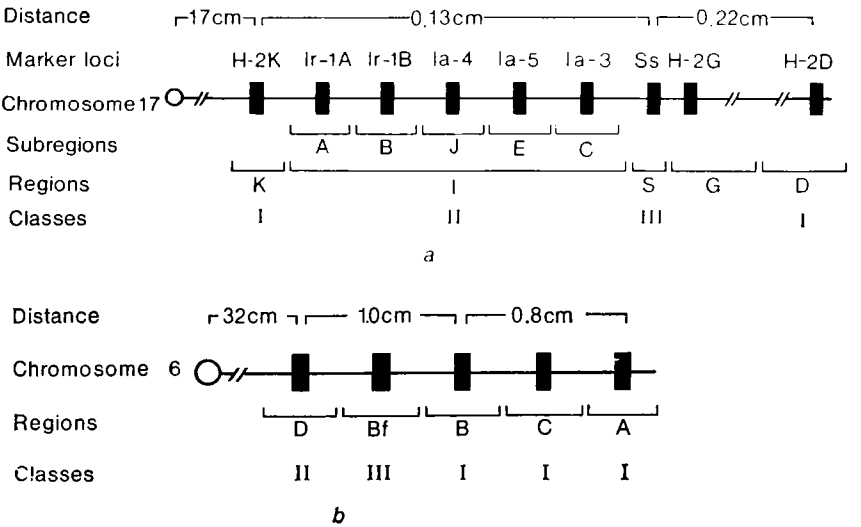



**Fig. 36.** Genetic map of histocompatibility gene system in mouse (H-2) (*a*) and in man (HLA) (*b*).

of immune response genes have been discovered by now. They control immune reactions to various, both synthetic and natural antigens. Practically all immune response genes are localized at one locus—at the chromosome site (in mice—chromosome 17, in man—chromosome 6). It is there that genes responsible for organ and tissue incompatibility in transplantations are also located (Fig. 36). This locus is termed the major histocompatibility complex (MHC) containing a total of over 1,000 genes. It may be considered as conclusively established that the immune response to all existing T-dependent antigens is under control of the specific Ir-genes localized in the MHC.

How do Ir-genes work? As early as 1966 it was shown by R. V. Petrov and co-workers (USSR) that the activity of Ir-genes is phenotypically expressed at the level of lymphoid cells populations. It turned out that the capacity to respond (or unrespond) to one antigen or another can be transferred to a lethally irradiated animal by means of lymphoid cells from a normal donor.

After T- and B-populations of lymphocytes had been discovered a question arose: at which particular cells of the immune system do

Ir-genes express their activity? First of all, it was established that thymectomy in new-born mice of different strains levels down the differences in the immune response to (T, G)-A-L. It should be pointed out that the activity of Ir-1-genes is expressed in a secondary immune response. After priming with (T, G)-A-L, mice of various strains produce practically equal amounts of IgM-antibodies. However, on a second immunization with (T, G)-A-L these antibodies are produced only in organisms of responding animals. The synthesis of IgG is to a larger extent a T-dependent process than the formation of IgM. Unresponding and thymectomized responding mice manifest the same response to (T, G)-A-L accompanied by the production of exclusively IgM-antibodies.

The genetically determined inability for an immune response in unresponding genotypes may be corrected by immunization with an antigen attached to an immunogenic T-dependent carrier, e.g., (T, G)-A-L to methylated bovine serum albumin (BSA). Unresponding animals immunized with a (T, G)-A-L and BSA complex produce antibodies to (T, G)-A-L. This, however, occurs only if animals unresponding to (T, G)-A-L exhibit a powerful genetic response to BSA. It points to the fact that the organisms of individuals unresponding to particular antigens possess necessary clones of B-cells potentially capable of specifically responding to the antigen. No response is elicited because of the absence of the T-helper function. In other words, Ir-genes may express their action at the level of T-cells.

The convincing evidence of the expression of Ir-genes at T-cells has been provided by resourcefully staged elegant experiments on so-called tetraparental (allophenic) chimeric mice. These chimeras are obtained by fusing two different mouse embryos at the stage of a 8-16-cell blastula. This is accompanied by the reimplantation of the new mosaic blastula in the uterus of a pseudopregnant female. The neonates, apart from genuine parents, have still another parent, a maternal one, who bears and nurtures them. If one fuses embryos, say, of a white and dark colour, the chimeric mice would have a zebra colouration. If one fuses embryos of the genotypes showing a high and a low level response to (T, G)-A-L, then in allophenic mice T- and B-cells of the high- and low-responder genotypes will coexist in common surroundings. To put it another way, B-cells of any genotype may interact with T-cells of a high-responder genotype. It has been demonstrated by H.O. McDevitt and co-workers that B-cells of a low-responder genotype in the presence of T-cells of a high-responder genotype perfectly well synthesize antibodies to (T, G)-A-L.

Consequently, Ir-genes express their action through a system of T-lymphocytes. Ir-genes may be differently represented at T-cells of different individuals. In contrast, the size and antigenic specificity of B-cell clones are identical in different genotypes. The

capacity to respond or unrespond to some particular antigen is limited by the representation of Ir-genes on T-cells. It has been demonstrated in recent years that the Ir-gene activity may be expressed on macrophage cells as well. In this case the role of Ir-gene products represented on macrophages in the form of a special protein (so-called Ia-proteins) reduces to the formation of an immunogenic activator. The latter is able to stimulate T-cells, carriers of the same Ir-gene. Differently speaking, Ir-genes are expressed at the surface of T-cells and macrophages so that T-cells can identify an antigen only complexed to autologous Ia-molecules of macrophages.

We have already reported that the crucial stages of immunogenesis are the processes of migration of stem cells and B-lymphocytes from the bone marrow and of T-lymphocytes from the thymus. These processes are primarily responsible for cooperation of T- and B-cells in the immune response. Viewed in this context, the role of the genotype in the expression of the aforementioned stages of immunogenesis is of much importance.

We already know that 1.5-2% of stem cells of the bone marrow are constantly coming to the circulatory system. These figures increase by a factor of manifold upon the administration of vaccines and other antigenic materials. It has been shown by Petrov and Khaitov that immunization with some particular antigen differently affects stem cell migration from the bone marrow depending on the mouse genotype. It turned out that in animals exhibiting a high level of response to antigen immunization with the same antigen brings about enhanced migration of stem cells. On the other hand, in a mouse strain showing a low response level to the same antigen, immunization, in practical terms, produces no effect on the intensity of stem cell migration and may even lower it. Consequently, a change in stem cell migration may play a definite role in the expression of genotypic differences of antibody production as one of the stages of immunogenesis at which genetic control of the immune response is expressed. It has been demonstrated that the process of stem cell migration is controlled by thymus-derived cells. Therefore one may not rule out a likelihood of the Ir-gene action on the migration reaction of stem cells following immunization. This action may be mediated by T-cells.

The fact that in mouse strains exhibiting a sluggish response to some particular antigen the processes of T-B cooperation are expressed weaker than in mouse strains with a high-response level to this antigen is also attributable to the expression of Ir-genes on T-helper cells.

As a response to the immunization, T-cell migration from the thymus and B-cell migration from the bone marrow likewise change. It appeared that in mice powerfully responding, say, to sheep erythrocytes (SRBC), upon immunization with this antigen, T- and

B-cell migration from the sites of their origination to peripheral lymphoid organs distinctly increases. Yet in mouse strains with a low response to SRBC the case is different. Specific immunization does not enhance T- and B-cell migration or, if it does, then only insignificantly. It has been established that the processes of lymphocyte migration are under control of the MHC genes.

Thus, genetic control over immunogenesis is expressed at this stage of lymphoid cell migration as well. A clear-cut effect of the Ir-genic control over T- and B-lymphocyte migration under conditions of the immune response has been established.

**Phenotypic correction of the immune response—search for new approaches.** The production of vaccination materials able to combat any infectious diseases has long been a cherished dream of immunologists. During many decades the notion of "low immunogenicity of the immunization preparation" were haunting the researchers and it was just the preparation that they were focussing on. Studies were conducted of the preparation's antigenic composition, search was undertaken for the most competent (protective) antigen coupled to procedures for its isolation, purification and synthesis. In many instances this line of endeavour failed to lead to advance. The immunized organism "refused" to develop an effective immune response despite the use of a highly purified protective antigen.

Immunogenetics shed new light on the problem of weak immunogenicity of antigens. The abstract terms a "strong" or a "weak" antigen are far from enabling one to gain an insight into the problem. In most cases, as has already been reported, what is implied is not a strong or weak antigen, but rather a high-responder or a low-responder organism. As an extreme expression of this the following rule may be formulated: there exist neither strong nor weak antigens, but high-responder and low-responder genotypes. It is for this reason that a need arose in new approaches to stimulating the immune response. They had to concern not only the antigen to be administered but also the responding system in order to find ways of converting low-responder individuals to high-responder ones. To achieve such a phenotypic correction artificial immunogens of a new type have been developed. These are constructed by combining the necessary antigen or antigenic determinant with a synthetic polyelectrolyte providing adjuvanticity, T-independence and (since Ir-genes express their activity through a T-lymphocyte system) Ir-independence of the entire macromolecular complex. This novel immunologic approach to the development of strong immunogens, anticipating vaccines of the future, was formulated in 1978. It was based on the studies in immunogenetics and on the findings summed up above. It consists essentially in bypassing the gene control over the potency of the immune response and making low responders (or non-responders) to the given antigen respond phenotypically high thus develop-

ing the immune response in full. Such a phenotypic correction opens up new, previously non-existing possibilities for the preparation of vaccinating materials. These would be used to counter as yet unconquered diseases and, possibly, to do away with cancer and allergies.

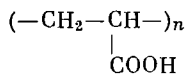
### The Effect of Synthetic Polyelectrolytes on the Immune Response

Many researchers in different countries are looking for and studying substances that stimulate the immune response. As a result a new discipline has emerged, immunopharmacology. Formerly immunologists evaluated the effect of the substances on the immune response solely from the amount of antibodies produced, i.e., from the ultimate stage of immunogenesis. Now the investigations have reached a qualitatively new level. We are looking for the immunostimulating substances proceeding from the mechanism of their action at the level of individual cellular events, individual steps of immunogenesis that constitute the immune reaction as a whole.

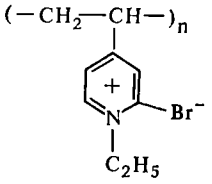
What are the implications of this approach? As has been pointed out earlier, the principal characters of the immune system are the two lymphocyte types. These are T- and B-lymphocytes, antibodies being products of the latter. Yet in most cases B-lymphocytes are uncapable of producing antibodies unaided, without the help of T-lymphocytes. They both must act jointly or, as immunologists say, cooperatively. T- and B-lymphocytes occupy various sites of the immune system. This fact, however, does not prevent their cooperation. T- and B-cells constantly migrate, staying at the sites of antigen accumulation. Here they cooperate to produce antibodies. Thus without the cellular migration and cooperation no antibody production would be possible.

It is these findings that provided a starting point for a working hypothesis stating that the substances stimulating the migration and cooperation of T- and B-lymphocytes should also stimulate the immune response as a whole. To verify this hypothesis many experiments were conducted using specially designed model systems to investigate different substances: nucleic acids, hormones, extracts of some plants, blood coagulants, synthetic polymers. The investigations done pointed to the feasibility and promise of the proposed approach. Substances were identified and studied that affected certain developmental stages of the immune response. Special attention was focussed on chemically comparatively simple synthetic polymer compounds, polyelectrolytes. These polymers have long been known to chemists. Their principal property consists in the following. In aqueous solutions their macromolecules acquire multiple electric charges, i.e., convert to polyions. A polyion, carrying a multiple negative charge is said to be a polyanion. Polyanions are

formed in electrolytic dissociation of polyacids and their salts. A polyion carrying a multiple positive charge is said to be a polycation. Polycations are products of dissociation of polybases and their salts. It was established in the last decade that many synthetic polyelectrolytes of most diversified structures, not being antigens, are capable of actively affecting individual developmental stages of immune response. Below are given structural formulae of some polymers of this class studied in detail at the laboratories of the present authors.



Polyacrylic acid (PAA)



PVP quaternary salt (PVP-$R_2$)

In the above formulae, the subscript $n$ (termed the degree of polymerization) indicates how many times the fragments shown are repeated in the polymer chain. These recurring fragments are called monomer links of a macromolecule. In immunologic experiments use is commonly made of polyelectrolytes with degree of polymerization (i.e., values of $n$) from tens to thousands.

The simplicity of synthesis and the definite structure of polyelectrolytes, the possibility of extensively varying their chemical composition, molecular weight (i.e., the degree of polymerization) and charge density on macromolecules open up much promise for their use in medicobiological, and, particularly, in immunologic studies. How is the effect of non-immunogenic polyelectrolytes on the immune function exerted?

**Stem cell-, T- and B-lymphocyte migration.** Mention has already been made that an important property of stem cells is their ability of migration from the bone marrow to the blood stream and of recirculation. It should be recalled that in 1972 Petrov and Khaitov elaborated an experimental model to study the action of various agents on the stem cell migration. As mice were lethally irradiated, a portion of the bone marrow was screened (part of the hind limb, generally at the level of half the shank). After 8-10 days following the irradiation, the number of haemopoietic colonies in the spleen was determined. Each of the colonies forms from a stem cell that has penetrated into the spleen from the screened portion of the bone marrow. The administration to the irradiated mice of a material influencing the stem cell migration tells on the number of colonies in the spleen. Differently speaking, the enhancement of the stem cell migration evokes an increase in the number of splenic colonies

and, on the contrary, the inhibited migration results in a drop in their number.

It turned out that PVP with a molecular weight of 10,000 daltons and PAA with that of 50,000 and 80,000 daltons enhanced the stem cell migration from the bone marrow threefold and more. In order to study the effect of polyelectrolytes on the migration of B-lymphocytes from the bone marrow and of T-lymphocytes from the thymus, special experimental models were elaborated.

A model was worked out to study the migration and proliferation of B-cells from the bone marrow using lethally irradiated mice with a portion of their bone marrow screened. After such a treatment the only source of proliferating B-cells remained the screened portion of the bone marrow. During first 7-10 days after the irradiation, the mice practically possess only B-cells proliferating from the screened area, hence the name B-mice. One can judge of the B-cell migration to the spleen from the ultimate accumulation of antibody-forming cells (AFC) after the administration of a standard quantity of T-cells to the "screened" individuals. Thus, upon a transfer of a definite dose of T-cells to the irradiated "screened" mice, the increase or decrease of accumulated AFC will be governed only by the intensity of repopulation of B-cells to the spleen. In experiments intended to study the effect of different substances on the B-cell migration from the bone marrow, 2 days after the irradiation standard doses of thymus cells and antigens (sheep erythrocytes) were administered to mice. After another 8 days the number of AFC was determined. The change in the intensity of the B-lymphocyte migration from the bone marrow to the spleen upon the administration immediately after the irradiation must, correspondingly, affect the quantity of B-lymphocytes (a variable) that interact with T-cells whose quantity is predetermined in the experiment (a constant). This change must affect the ultimate quantity of AFC as well. It appeared that the administration of PAA, PVP and other related polyelectrolytes markedly enhances the B-lymphocyte migration from the bone marrow. Moreover, polyelectrolytes do this three to four times as effectively compared to natural stimulants.

We have developed an experimental model to study the T-helper cell migration and also to evaluate the action of various materials and their effect on the processes of T-cell dissemination. In lethally irradiated mice whose thymic area was screened there occurs repopulation of peripheral lymphoid organs by T-cells migrating from the thymus. During first 24 hours after the irradiation they can be practically considered T-mice. Therefore the immunization with a thymus-dependent (TD) antigen of irradiated mice that had the thymus screened brings about an insignificant accumulation of AFC in the spleen. A transfer to such hosts of B-cells is accompanied by a pronounced increase in the immune response due to the cooperation of

B-cells with thymus cells that have repopulated the spleen. Since the accumulation of AFC in the spleens of irradiated mice that have received a standard dose of B-cells is directly related to the number of T-helper cells, a change in the rate of thymocyte migration leads to a change in the cooperation effect and, correspondingly, tells on the level of the immune response. Thus, if the lethally irradiated mice have only the thymus screened and are administered a limited dose of B-cells, then the T-cell migration would be a variable quantity that can be evaluated from the level of the immune response. In other words, the accumulation of AFC following the immunization with TD antigens in the given model is entirely governed by the rate of T-cell migration from the thymus.

In order to study the effect of polymers on the T-lymphocyte migration, mice were lethally irradiated, the thymic area being screened. Then the material under test was administered to the mice. Two days later they received a standard dose of bone marrow cells (BMC) and sheep erythrocytes (SRBC), and eight days thereafter the number of AFC in the spleen was determined. With the administration of one preparation or another immediately after the irradiation, the immune response can only change owing to its effect on the T-cell migration from the screened thymus. It appeared that polyelectrolytes essentially stimulate the T-cell migration from the thymus.

**Cell-to-cell interaction in immunogenesis.** As has been pointed out, the immune response of an organism is a result of a multistep interaction of the immune system cells. What effect do non-immunogenic polyelectrolytes exert on this interaction?

*Macrophage-to-lymphocyte interaction.* The macrophage-to-lymphocyte interaction necessary to induce the immune response may be achieved by a direct contact. A. Rosenthal has proposed a technique to evaluate such an interaction. The *in vitro* incubation of lymphocytes with macrophages gives rise to the formation of what is called clusters. These are accumulations of lymphocytes around macrophages. The physiological significance of such a form of the macrophage-to-lymphocyte interaction, if the macrophage's partner is a thymus cell, is its effect on the maturation of immature thymocytes. It occurs when the latter migrate through the corticomedullar area of the thymus to the medullar area (i.e., macrophage-rich areas) where thymocytes mature. Therefore the stimulation of contact interaction of macrophages with immature lymphocytes of the T-line is of much theoretical and practical interest.

It emerged that an addition of synthetic polyelectrolyte into the system of interacting macrophages and thymus cells enhances the contact interaction of these cells. Polyelectrolytes assisted the macrophage-T-cell cluster reaction even in such cases when this reaction should not have occurred due to the genetic incompatibility of donors

of T-cells. It is intriguing that T-cells separated from macrophages are endowed with a considerably enhanced helper activity if they were "cemented" with polyelectrolytes.

*T-B cooperation.* We are already aware of the fact that for cells that synthesize antibodies (AFC) to form, the cooperation of T- and B-lymphocytes is required. Tests to study the effect of synthetic polyelectrolytes on T-B cooperation were carried out using "live test-tubes"—mice that had received a lethal dose of penetrating irradiation. As is known, upon the irradiation it is the immune system cells that die first. For this reason, the organism of an irradiated animal in fact loses the ability to produce antibodies in response to the administration of an antigen. This ability, however, can be reconstituted using T- and B-cells from genetically compatible donors. This is actually the way the numerous experiments have been staged. The results of some of them are presented in Table 13.

**Table 13.** Enhancement of T-B-Lymphocyte Cooperation under Effect of Synthetic Polymer Adjuvants

| Polymer adjuvant | Mol. weight of polymer, daltons | Number of AFC in spleen |
|---|---|---|
| None | — | 260±30 |
| Poly-4-vinylpyridine (PVP) | $1 \cdot 10^5$ | 1,300±250 |
| Poly-2-methyl-5-vinylpyridine (PMVP) | $(8\text{-}10) \cdot 10^4$ | 1,200±100 |
| Polyacrylic acid (PAA) | $8 \cdot 10^4$ | 800±100 |

*Note.* Following a lethal dose of irradiation mice were given a mixture of T- and B-lymphocytes and sheep erythrocytes. The dose of adjuvants was 50 mg/kg.

The irradiated mice received definite amounts of B- and T-lymphocytes and then were immunized with sheep erythrocytes (an antigen). After a while the quantity of AFC in the spleens of the immunized mice was determined. This number is indicative of the effect of T-B cooperation. If mice receive only T-lymphocytes with an antigen or only B-lymphocytes with an antigen, no AFC are produced. A significant amount of AFC is produced only upon the administration to irradiated mice of a mixture of T- and B-cells which is indicative of the effect of T-B-lymphocyte cooperation.

As can be inferred from Table 13, if animals that have received a mixture of T- and B-lymphocytes and an antigen are administered a polymer stimulator (adjuvant), the number of AFC will markedly increase. Differently speaking, under the action of polymer stimulators (both polybases and polycarbon acid) the effectiveness of T-B

cooperation significantly increases. It should be emphasized that monomers that make up the given polymer compounds produce no effect on the formation of AFC in the spleens of the recipient mice that have received the T- and B-lymphocyte mixture.

Of considerable interest are findings on the dependence of adjuvanticity on the length of the chain (the degree of polymerization $n$) of polymer adjuvants. So, after using narrow fractions of a PVP salt as an adjuvant, it turned out that chains of this polycation 100-200 links long, much like an analog of a monomer link, 4-ethylpyridinium bromide, do not as yet possess an appreciable capacity to augment the immune response. With the degree of polymerization equal to 1,000 the stimulating action is markedly expressed. As the polycation extends, this action insignificantly enhances. One should not, however, consider $n = 1{,}000$ to be a "magic" figure. Depending on the nature of the polyion, the critical value of $n$ at which a polyelectrolyte becomes an immunostimulant may vary over a very wide range.

**Substitution of T-helper function.** As the mechanism of the effect of synthetic polyelectrolytes on immunogenesis was studied, one of the most remarkable facts emerged from experiments in so-called T-deficient mice, or B-mice. The administration of antigens (e.g., sheep erythrocytes) to such animals elicits no immune response. Antigens of sheep erythrocytes are thymus dependent, so in the absence of T-helpers the immune systems of the animals cannot be triggered.

Things are different in the case of immunization with sheep erythrocytes followed by practically simultaneous administration of PAA or PVP. In this case B-mice develop a clear-cut immune response. The amount of AFC in the spleen upon the administration of PAA increases almost 17-fold, upon the administration of PVP almost 27-fold.

It might seem from the aforementioned data, that the presence of polymer adjuvants compensates, at least partially, for the lack of T-helpers necessary to induce antibody genesis. The investigated polyelectrolytes stimulate a T-independent immune response to the administration of a typical T-independent antigen.

As has been reported, one of the crucial T-helper functions is to give the B-lymphocyte a second non-specific signal. Therefore one may assume that this signal in the particular case is somehow ensured by a polyelectrolyte which directly or indirectly cooperates with B-lymphocytes. The actual mechanisms that give a second signal are not exactly determined. However, the assumption of a non-specific activation of B-lymphocytes by linear polyelectrolytes that adhere to them is in agreement with the findings obtained from both *in vitro* and *in vivo* experiments. These findings will be reported in what follows.

**Polyclonal activity.** Attempts had been made for quite a number of years to develop such methods of cultivating lymphoid cells that would make it possible to initiate an immune response by *in vitro* antibody production. R. Mischell and R. Dutton first achieved this in 1966 for a splenic cell culture. The previous methods had permitted the *in vitro* antibody synthesis only by cells removed from an immunized animal. In contrast, R. Mischell and R. Dutton succeeded in creating such culture conditions under which the entire process, from the reception of the antigenic stimulus to the antibody formation, occurred *in vitro*. Their method enabled them to reveal formerly unknown mechanisms of antibody genesis, to detect molecular mediators of lymphocytes, etc.

When studying the action of synthetic polyelectrolytes *in vitro*, it was first established that they exhibit their stimulating activity towards antibody genesis in a culture of splenic cells immunized with antigen *in vitro* as effectively as *in vivo*. In further studies it was essential to answer the question concerning the way polymers affect lymphocytes by themselves, without antigenic stimulation. It should be noted that even prior to a specific immunization it was possible to find in lymphoid tissues AFC specific to the given antigen (so-called background AFC). Their number is relatively small. Usually this quantity is 1-10 per 10 million lymphoid cells. After the effective immunization considerable amounts of AFC are produced specific to the antigen administered to an organism or a culture *in vivo*. The amount of accumulating "immune" AFC following the immunization increases in a matter of a few days to 500-5,000 for a million of lymphoid cells.

In *in vitro* cultures containing no antigen, splenic cells treated with a polyelectrolyte (PAA polyanion or polycations, various PVP salts) produced definite amounts of cells that synthesized specific antibodies against different antigens (erythrocytes of sheep, donkey and horse, trinitrophenyl hapten, etc.). The *in vitro* experiments have also demonstrated that the administration to mice of PAA and other polyelectrolytes without an antigen evokes the development of AFC that synthesize antibodies to various antigens. Thus the so-called phenomenon of polyclonal activation of B-lymphocytes with non-immunogenic synthetic polyelectrolytes was established. This phenomenon consists in the non-selective induction of the conversion of various B-lymphocyte clones of AFC directed against a whole array of antigens that were not previously administered to the organism.

The phenomenon of polyclonal activation of B-cells is known for a number of bacterial macromolecular structures carrying negative charges (lipopolysaccharides, dextrans, levans, and their derivatives). For example, the direct stimulation of B-cells with *E. coli* lipopolysaccharide causes the proliferation of different B-cell clones

followed by the production by the latter of antibodies showing a vast range of specificity. Synthetic polyelectrolytes, compared with lypopolysaccharides, are weak polyclonal activators. They increase the number of background AFC 3-4 fold. The polyclonal activity of such polyelectrolytes is associated with their direct effect on B-cells, with no other cellular systems, in particular, T-system of lymphocytes, participating. So, the administration of PAA or PVP derivatives to normal mice, B-mice or nude mice is in all cases accompanied by an increase in the number of background AFC to different antigens 3-4-fold.

It has been likewise shown by *in vitro* experiments that PVP and PAA significantly increase the cell division rate. To elucidate the nature of cells that respond by division to the administration of synthetic polyelectrolytes, a study of their action on fractionated T- and B-lymphocyte populations in the *in vitro* culture was undertaken. It emerged that under the action of PAA solely B-cells start to divide and to intensely synthesize DNA. The T-cell culture showed no appreciable response to PAA. Polycations, however, enhanced both B- and T-cell division.

It has been demonstrated that the activation of lymphocytes using synthetic polyelectrolytes involves the cellular ionic homeostasis. It is expressed in a marked increase of the membrane permeability to ions (for details refer to what follows). The change in permeability is apparently attributable to some membrane structural alterations. These may include, e.g., the formation of protein aggregations or phase transition of membrane matrix lipids. A drop in the membrane potential may be an important factor in the mechanism of the change of permeability. A natural consequence of the increased permeability is the accumulation in the cytoplasm of sodium and calcium ions. The intracellular gradients in the cation concentration, in particular that of calcium cations, may serve as a triggering mechanism in the control over enzyme activity and in the modification of the cellular metabolic systems. It also suggests that it is possible to artificially control the processes of metabolism, growth, division and differentiation of lymphocytes. It may be done by modifying these or other characteristics of the system of ionic homeostasis. Proof to support this last assumption comes from the familiar fact of inclusion or blocking of lymphocyte division by means of a calcium or potassium ionophore, respectively.

**T-suppressor activity.** Mention has already been made of the important role played in the control over the immune response by T-suppressor cells. Under the normal conditions these natural regulators inhibit immune reactions preventing them from developing beyond limits. It turned out, however, that sometimes T-suppressors play an apparently negative role interfering with the normal course of the immune response. Thus, definite mouse strains are known to lack

the capacity to manifest a normal immune response to some antigens. This is connected with the enhancement of the activity of T-suppressors whose function is under control of immune suppression genes, Is-genes. Some immunodeficiency-related diseases of man are known that are also attributable to the enhanced T-suppressor activity. Practically in all cancer types and many infectious diseases (viral, bacterial, parasitic) the increased activity of these cells inhibits a high immune response. All this strongly suggests the importance of being able to lower or to temporarily abolish T-suppressor function.

If one recalls that synthetic polyelectrolytes ensure the T-independence of the immune response to trivial T-dependent antigens due to the capacity to directly trigger B-cell reproduction followed by their conversion to AFC, it would seem quite reasonable to try to use such polymer stimulants for these very ends. We used a model system according to the following protocol. Normal mice were primed with a very high dose of antigen. A similar action evokes a selectively expressed T-suppressor activity. For this reason, upon repeated immunization with the same antigen no immune response is brought about. Naturally, should T-suppressors be taken from suppressed animals and these cells be grafted to normal, genetically identical (syngeneic) recipients immunized with an optimum immunogenic dose of antigen, then the immune response of such recipients would be inhibited. It appeared that administration of PVP or PAA reconstitutes the immune response that was inhibited by T-suppressors.

Thus, synthetic polyelectrolytes abrogate the effect of T-suppressor cells. Since targets for T-suppressors are provided by T-helper cells, one of likely mechanisms of such an effect may be the direct stimulating action of polymers on B-cells. This is consistent with their capacity to substitute for the T-helper function and to directly evoke the B-lymphocyte activation.

**Antigenic competition.** The phenomenon of antigenic competition was first reported by L. Michaelis in 1904. An administration of alien serum to rabbits brought about the generation of antibodies directed against the globulin but not the albumin. The injection to animals of solely the albuminous fraction of serum evoked the production of antibodies to the albumin. The phenomenon of antigenic competition is thus expressed in the inhibition of the immune response to one antigen or antigenic determinant. This inhibitory action is associated with the introduction of an alien antigen or determinant into the organism. Taking into account the antigenic competition is of important practical value. It is especially so viewed in terms of vaccination where microbial or viral antigens are administered concurrently (polyvalent vaccines) or successively.

One distinguishes between intramolecular and intermolecular forms of antigenic competition. The former involves the presence of various competitive antigens or determinants on one immunogen mol-

ecule, e.g., Fab- and Fc-fragments on one IgG molecule of rabbit. The administration to mice of rabbit IgG induces the generation of antibodies to the Fc-fragment. However, the immune response to the Fab-fragment proves to be suppressed. The immunization of mice solely with a Fab-fragment evokes a pronounced anti-Fab response. The intermolecular competition is observed when competing antigens are present on different molecules. For example, the simultaneous administration of a mixture of Fc- and Fab-fragments obtained from papain-digested rabbit IgG leads to the inhibition of the immune response to the Fc-fragment. A variety of intermolecular competition is that expressed upon the administration of antigens at different times (successive competition). For example, upon the successive administration at a four-day interval of sheep erythrocytes (SE) and then horse erythrocytes (HE) the suppression of antibody production to HE occurs. The antigen inducing the suppression of the immune response is said to be a dominant antigen.

It has been demonstrated that T-suppressors might be the cells that are responsible for antigenic competition. So, R. K. Gershon and K. Kondo found out in 1971 that B-mice exhibited a T-independent IgM response to HE that is not suppressed by a preliminary injection of SE. An additional administration of T-cells to B-mice increases drastically the immune response to HE. The higher T-dependent response is inhibited by a preliminary injection of SE.

In practical terms the phenomenon of antigenic competition is of great importance in the immunization by antigenic mixtures, e.g., polyvalent vaccines. To achieve advance in the preparation of synthetic polydeterminant vaccines, each intended to evoke an immune response to a particular predetermined antigen, also calls for a solution of the problem of antigenic competition. Therefore an attempt to control the effect of antigenic competition with an intent to abrogate it is of much interest. Our laboratory has investigated the action of the polyanion of acrylic acid and N-vinyl pyrrolidone copolymer on the effect of successive antigenic competition. In order to induce the antigenic competition mice were administered with HE and SE, the dose being $1 \cdot 10^9$ (dominant antigen). Four days thereafter the animals were immunized correspondingly with erythrocytes of a different species, the dose being $2 \cdot 10^8$ of SE or HE (immunizing antigen). After another 4-5 days the amount of AFC in the spleens was determined.

The chosen corpuscular antigens, HE and SE, are in fact antigens that are not cross-reactive. A preliminary injection of HE before the immunization with SE evokes a 4-fold suppression of the immune response to the immunization antigen. The case was the same in an immune response to HE upon the pre-immunization with SE. This effect is governed by the nature of the dominant antigen because a preliminary injection of a T-independent antigen, poly-N-vinyl pyr-

rolidone (mol. weight 360,000 daltons) does not lead to a drop in the number of antigen-specific AFC. If experiments were carried out on mice thymectomized 30 days or more before the experiment, no antigenic competition was observed either. Consequently, the phenomenon of the inhibition of the immune response to an antigen following the administration of unrelated antigens is thymus-independent. These findings are in good agreement with the assumption of the participation of short-living non-specific T-suppressors in eliciting the successive competition phenomenon.

We have shown that the administration of a polyanion at the moment of injection of a dominant antigen induces the abrogation of the immune response suppression. This abrogation is attributable to the T-independence of the immune response including the independence of T-suppressors. Another likely cause may be the inhibition of the T-suppressor function.

**Immunostimulant activity.** The reported data provided experimental proof of the possibility of a directed action upon the level of the immune response. It can be achieved by varying the migration of stem cells, T- and B-lymphocytes and T-B-cell cooperative interaction. It has been shown that synthetic unnatural polyanions and polycations, as a result of the enhancement of cell migrations and T-B-cell cooperation, lead to the substitution for the helper function of T-cells, evoke the B-cell activation and T-suppressor cell inactivation. It would be logic to expect that these compounds should exhibit immunostimulant properties when acting on different animals and inducing various types of the immune response.

*In various animal species.* The administration of PVP or PAA to mice and rats immunized with SE brings about a 4-6-fold increase in the immune response.

The dose-effect dependence is observed upon an injection of various doses of polyanion (copolymer of acrylic acid and N-vinyl pyrrolidone) to SE-immunized dogs. As is seen, an increase in the polyanion dose causes the enhancement of AFC generation. Upon an injection of this polyanion concurrently with the immunization using tetanus anatoxin, the antibody production compared with control (vaccination only) enhanced. In dogs it increased more than 20-fold, in rabbits 4-fold, in rats 20-fold. On the other hand, its injection together with a whooping-cough vaccine enhanced the antibody production: 12-fold in dogs, 260-fold in rabbits, and 10-fold in rats.

*In low-responder genotypes.* It has been pointed out that genetic differences in the level of the immune response in animals of different genotypes are proponderantly manifested at the level of T-cells. It emerges that a lower accumulation of AFC in mice of low-responder genotypes compared to high-responder individuals is due to the weakly expressed T- and B-lymphocyte migration and cooperation upon the immunization with a particular antigen. Consequently, there

was every ground to assume that the immune response in animals of low-responder genotypes could be enhanced. It can be achieved by the administration of polyelectrolytes that stimulate these processes. In all the experiments an injection of PVP, PAA and some other polyelectrolytes led to a higher immune response in the case of low responsiveness to one antigen or another (anatoxins of agents of gas gangrene, sheep erythrocytes, conjugates of haptens and proteins). In so doing, the level of the immune response in low-responder genotype individuals that received, concurrently with an antigen, polymers reached the level of the response of a high-responder genotype animals.

Thus, genetically determined deficiencies of the immune system localized at the level of cells that participate in the immune response can be corrected with synthetic polyanions and polycations.

All the aforementioned studies examined the impact of synthetic polyelectrolytes on the immune response to complex multideterminant antigens (erythrocytes, proteins, hapten-protein conjugates). As is known, the immune response to such antigens is controlled by many Ir-genes. Therefore, of much interest are the findings concerning the effect of PAA and PVP on the immune response to the artificial antigen (T, G)-A-L controlled by one gene Ir-1. It appeared that under the action of polyelectrolytes the immune response of low-responder animals attains the level of high-responder individuals that received no polymer, i.e., there occurs a phenotypic correction of a low immune response to (T, G)-A-L. In other words, the level of immune response of animals that are genetically low responders to (T, G)-A-L becomes phenotypically similar to the level of high-responder individuals.

*In T-deficient animals.* The regularities of genetic control over immunity have been described and the principle of unambiguity of the immune response has been formulated above. This type of genetic control is characterized by specificity. The same individual may be a high-responder to one antigen and a low responder to another one. This is what occurs to a healthy organism. The essence of all it is genetic polymorphism. At the same time immunologic "weakness" may also be of a non-specific nature, many or all antigens being involved. However, in this case the genetic distinctions of individuals are not ruled out either. Usually defects, immune system diseases, concern separate links of the immune system while the remaining links are allowed to function. Due to this, the "weakening" of the immune system is not a uniform depression of the activity of the entire immune system but rather a form of one or another level of its organization, most frequently, of its T-system.

Consider the defects of the immune system in more detail. We will start with congenital (or primary, as they are sometimes termed). Children born with immunologic defects may appear healthy in the

first weeks of their life, as long as in their blood circulate antibodies received in the mother's uterus and with the early maternal milk. Yet very soon the latent disorders reveal themselves. Ceaseless diseases ensue: pneumonia, abscesses on the skin, maxillar sinusitis, otitis, etc. Moreover, they are liable to recur. It would be more correct to describe congenital immunodeficiencies a primary since such a name suggests immunologic primacy of all events. In other words, disease, delayed growth and development, death result from the primary cause, inborn immunodeficiency. It is customary to describe as secondary the acquired defects of the immune system. These may originate, for example, upon exposure to ionizing radiation that leads to radiation injury. Immunity is dramatically depressed because the lymphoid tissue—immunity organ—is destroyed by the irradiation. Secondary defects develop following prolonged starvation, infectious disease, ageing.

The primary immunodeficiencies are associated with genetic disturbances in the development of either T- or B-cells, combined defects frequently occurring. The cause of most secondary immunodeficiencies is typically the "weakness" of the lymphocyte T-system.To put it differently, under many congenital and acquired in lifetime immunodeficiency conditions the developmental disturbances of the lymphoid system are localized at the level of T-cells. Consequently, use of polyanions and polycations to ensure the T-independence of the immune response opens up the way for stimulation of a T-deficient immune response in the case of diseases related to immunodeficiencies.

A most convenient model of an inborn T-deficiency is provided by congenially athymic nude mice. In them genes that control the formation and development of the thymus are linked with genes responsible for hair growth. Therefore the absence of hair is a marker of an inborn underdevelopment or total absence of the thymus. Nude mice are incapable of developing either a primary or a secondary immune response when immunized with standard T-dependent antigens, e.g., sheep erythrocytes. This is due to the absence of T-lymphocytes known to be formed in the thymus. An injection of a synthetic polyelectrolyte to nude mice results in a marked increase both in the primary (IgM-antibody production) and in the secondary (IgM- and IgC-antibody production) immune response to sheep erythrocytes. We anticipated this effect, since synthetic polyelectrolytes replace the T-helper function.

As has been pointed out, an injection of PAA and PVP to B-mice (animals with a total T-deficiency) ensures the production of fairly large amounts of AFC. In terms of their immune status B-mice are actually similar to nude mice. A milder defect in the lymphocyte T-system is evoked by thymectomy not followed by irradiation. Under such conditions the animals lose only a part of T-cells. Pubescent

thymectomized mice may serve as a model of a secondary T-deficiency. In this latter case the amount of T-cells in the organism decreases gradually. Immunization of thymectomized mice with a T-dependent antigen at different time intervals after the operation demonstrates that their capacity for an immune response begins to degrade a month and more after thymectomy. Upon administering such T-deficient animals with a synthetic polyelectrolyte, the capacity for immune response is completely reconstituted.

A marked T-deficiency develops with ageing. This is attributable to the age-induced thymus atrophy. It is lack of T-lymphocytes that is responsible for many diseases in aged people. It has been established that the administration of a synthetic polyelectrolyte (PVP polycation or polyanion—copolymer of acrylic acid and N-vinyl pyrrolidone) to old animals in which the capacity for antibody production is decreased by a factor of tens practically completely reconstitutes their capacity for immune response. What occurs is, as it were, a rejuvenescence of the old immune system.

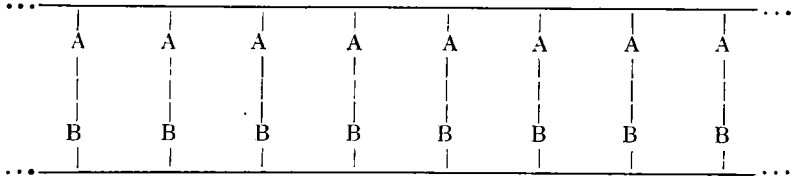
**Physicochemical principles of immunostimulant activity of polyelectrolytes.** Notwithstanding the substantial distinctions in the structure and even in the very nature of elementary chemical links of macromolecules of investigated polyelectrolytic stimulants, the immunostimulant activity of all these compounds is about the same in magnitude. All of them make for a manifold increase of the amount of AFC specific to the administered antigen. It is also essential that, as has been previously noted, monomer analogs of links of the aforementioned polymers do not produce any stimulating effect. Proceeding from these observations it is natural to conclude that the mechanism of polymer adjuvant activity is not directly associated with fine peculiarities of the structure of their links. It is also natural to suggest that immunostimulant activity is based on certain common mechanisms that must be primarily due to a number of common properties of macromolecules.

One of such properties is a capacity for the multisite cooperative interaction with other chemically complementary macromolecules followed by the formation of very stable interpolymer systems or for the multisite cooperative sorption on chemically complementary surfaces.

*Cooperative ability viewed in physical terms.* It is usual in molecular physics to describe as cooperative such systems in which the state of each of the set of their components is essentially dependent on the state of the adjacent components. Interaction of elements in such systems are said to be cooperative interactions, and the processes in which these interactions are expressed, are termed cooperative processes.

A linear macromolecule, as such, represents a cooperative system. It means that each of its links is liable to coordinate its behaviour

(e.g., rotation or spatial displacement) to that of the adjoining links. If two macromolecules are made up of chemically complementary monomer links (e.g., if one contains a sequence of donors and the other, of hydrogen bond acceptors; or, alternatively, if one contains a sequence of anionogenic and the other of cationogenic groups) then such a pair may form a cooperative complex of the type $(A \ldots B)_n$:




The $(A...B)_n$ polycomplex may be perfectly stable over a sufficiently large range of change of conditions (temperature, pH, ionic strength, etc.) even if the energy of a separate bond A...B is very small such that the corresponding complexes A...B or $A...B(B)_{n-1}$ in this interval are markedly dissociated. The additional stabilization of the interpolymer complex $(A...B)_n$ exists due to the fact that the energy necessary for disruption of one internal bond A...B is not compensated by an increase in entropy. This is because no additional translational degree of freedom appears in the system as in the case of rupture of a similar bond between the monomers A and B or between the monomer A and polymer $(B)_n$. For the disintegration of an interpolymer complex to begin it is necessary to simultaneously disrupt in it a few adjacent bonds, the required energy being concentrated on each of them. Other conditions being equal, this is a much less probable event than the accumulation of the required energy on one randomly selected bond. In contrast, under definite conditions (i.e., on attaining a certain value of temperature, pH, ionic strength, etc.) the transition of the interpolymer complex from a perfectly stable to a completely unstable state, that is, its practically total dissociation is realized over a very narrow interval. Occasionally it may occur almost in a "jump". Such transitions are said to be cooperative.

The essence of a system's cooperative stabilization can be inferred if we consider the behaviour of a long string of beads on a table that is constantly vibrating. The vibration simulates thermal motion. Were not the beads tied to form a chain, they would very soon fall from the table. The way the string behaves is different. Even though its separate segments are shifted, it will, however, much longer stay on the vibrating table. The cohesive force of an individual bead with the surface of the table is in both cases the same. Yet the beads, united as they are, cannot be displaced independently of one another. Therefore the probability of the entire chain losing cohesion with the table and thus falling down proves to be much less than for the same num-

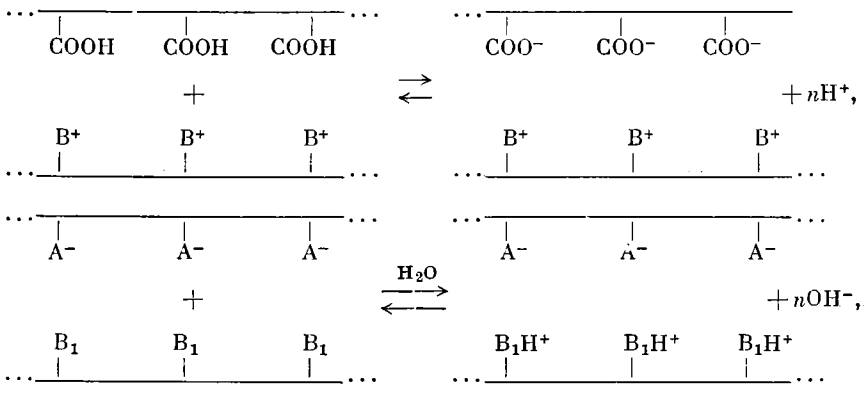
ber of separate beads. In contrast, if the vibration becomes so violent as to cause the string to fall from the table, all the beads will lose cohesion with the table practically at the same time, in a jump. Such a fall would to a certain degree simulate a cooperative transition.

A double helix of complementary chains of DNA is a classical example of a cooperative interpolymer complex, DNA denaturation being an example of a cooperative transition. Similar examples are legion. Among these are, in particular, interpolyelectrolyte systems made up of pairs of oppositely charged polyions. The reaction between linear polyelectrolytes can be schematically represented as follows:

$$\left|\begin{array}{ll} -AK & A_1B- \\ -AK & A_1B- \\ -AK & A_1B- \\ -AK & A_1B- \\ -AK & A_1B- \end{array}\right| \rightleftarrows \left|\begin{array}{ll} -A^- & {}^+B- \\ -A^- & {}^+B- \\ -A^- & {}^+B- \\ -A^- & {}^+B- \\ -A^- & {}^+B- \end{array}\right| + nK^+ + nA_1^-,$$

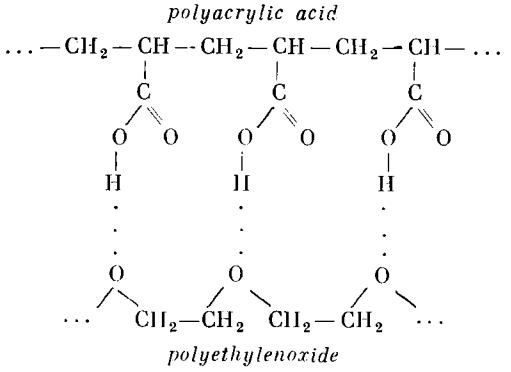
where AK is a link of polymer acid or its salt, $A_1B$ is a link of polymer base or its salt.

The reaction between links of initial polymer chains forms salt bonds. If one of polyelectrolyte components represents a weak polyacid (e.g., polycarbon acid) or a weak polybase, and the second, correspondingly, represents a salt of some polybase or polyacid (i.e., a strong polyelectrolyte), then the origination of complexes is accompanied by a release of protons or hydroxyl-ions

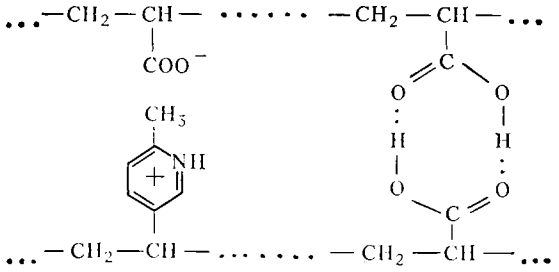



where $B^+$ is a charged basic group, e.g., a charged amino group; $A^-$ is an acid residue; $B_1$ is an uncharged basic group, e.g., a primary, secondary or tertiary amino group belonging to links of polymer chains. (To simplify matters, counterions of initial polymer salts are not shown.)

An example of cooperative interpolymer complexes in which the multisite interaction of components is achieved due to the formation of hydrogen bonds may be provided by a PAA (polymethacrylic acid) —polyethylenoxide system:




Interpolymer complexes are also known in which a multisite interchain bond system includes both salt and hydrogen bonds. Among these are PAA and CP-AA·VPD complexes



In this particular case the copolymer macromolecule in the polycomplex takes on, as it does, the role of a heterofunctional cooperative partner with respect to the PAA homopolymer.

Kabanov and co-workers did a detailed study of some water-soluble cooperative complexes of synthetic linear polyelectrolytes and globular proteins, in particular, blood serum proteins. The formation of such systems does not, as a rule, evoke protein denaturation. The role of a cooperative partner with respect to the linear polymer is played by the array of functional groups at the protein globule surface. In terms of an ability for multisite interaction the amphoteric protein globule surface is also heterofunctional. Bovine serum albumin (BSA), e.g., in neutral aqueous solutions gives rise to complexes

with polycations, with some polyanions and certain amphoteric linear polyelectrolytes. Depending on the nature and chemical structure of the linear partner chain, an essential contribution to the system's stabilization may be made, apart from salt bonds, by hydrogen bonds and hydrophobic interactions. The formation of complexes of proteins and linear polyelectrolytes consists actually in "sticking" of more or less extended segments of polyelectrolyte chains to the surface of protein globules. Depending on the degree of polymerization, other conditions being equal, a single macromolecule may "stick" either to one or a few globules (Fig. 37). It should be particularly emphasized that in the solution not all the links of a linear polyelectro-

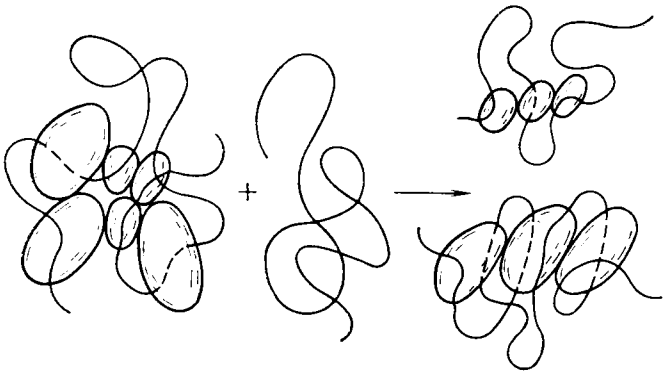


**Fig. 37.** Redistribution of different protein globules between linear polycations.

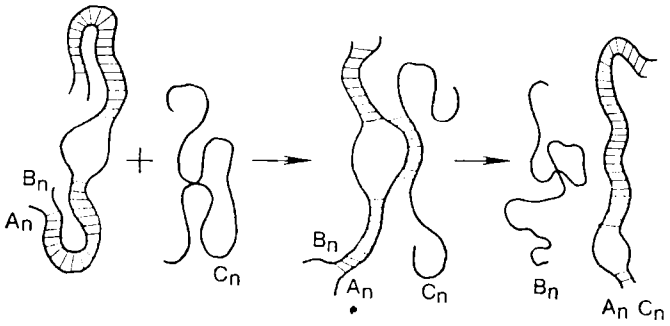
lyte chain loaded with protein globules "stick" to the latter. Sufficiently long free segments of the chain are preserved in the shape of loops or free termini that ensure a potential possibility of an additional multisite linkage of other objects.

*In search of an optimum.* The important property of interpolymer complexes established in Kabanov's laboratory is their ability to readily undergo a reaction of macromolecular substitution in a solution. Differently speaking, one of two chains already incorporated in an interpolymer complex $(A...B)_n$, e.g., $B_n$, can be substituted by another chain $C_n$. This may occur if the free energy of the formation of the bond A...C in the complex is several times lower compared with that of the bond A...B:

$$(A...B)_n + C_n \rightleftharpoons (A...C)_n + B_n$$

Such types of reaction seem to originate at sites of defects in the structure $(A...B)_n$ and pass through an intermediate stage of the formation

of triple complexes according to this scheme:




From the likelihood of easy occurrence of macromolecular substitution and exchange reactions the possibility naturally follows of intra- and intermolecular rearrangements of interpolymer complexes.

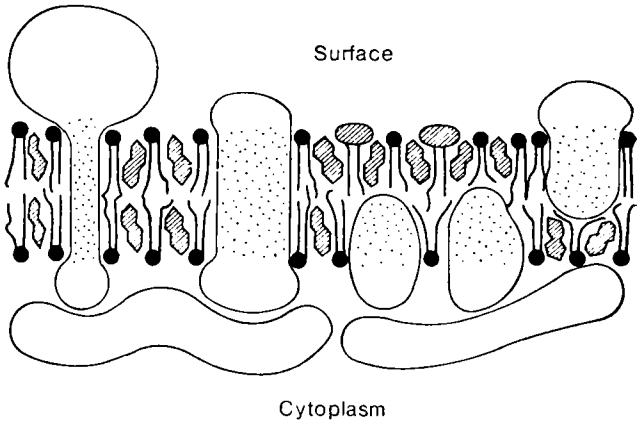



**Fig. 38.** Schematic presentation of cross-section of cell membrane.

These come up handy to seek conformations (or compositions) corresponding to deeper minima of the system's free energy.

Macromolecular exchange and substitution reactions in solutions are also typical of polyelectrolyte-globular protein complexes. It has been demonstrated, e.g., that the addition of PVP-$R_2$ both to aqueous solutions of artificial mixtures of serum proteins and to intact serum first gives rise to mixed interpolymer system. However, an increased amount of polycations in the system results in the distribution of individual proteins among separate polycations followed by the formation of individual soluble complexes (Fig. 38). The driving force of the distribution seems to be furnished by an additional "positive" interaction of like molecules within individual

particles of a polycomplex, e.g., owing to the likelihood of their more compact packing. It is of note that the onset of equilibria of a type shown in Fig. 38 occurs very rapidly, almost at a rate of displacement of reagents. Another example of the macromolecular substitution is quantitative displacement of serum proteins from their systems with PVP-$R_2$ in intact blood serum by polyanions of a natural polysaccharide, heparin. Such polyanions, due to the high charge density, exhibit a higher affinity to polycations compared with protein globules. The last of the examples cited suggest the selectivity of interaction or at least the likelihood of its expression under certain conditions. It has been previously shown by *in vivo* experiments that heparin quantitatively links linear polycations even in whole blood, i.e., under conditions of all serum proteins and hemocytes interacting with them.

The multisite adsorption of polyelectrolytes on colloid particle surfaces also refers to the category of such cooperative phenomena that are in general terms similar to the origination of interpolymer complexes. It is known, in particular, that polycations are firmly adsorbed on negatively charged colloid dispersion particles. The same is true for polyanions with respect to positively charged colloids. Should the length of polyelectrolyte chains be sufficiently large (i.e., the likelihood of adsorption of different segments of the chain on surfaces of two different particles is sufficiently large), then colloid particles cohere. The latter phenomenon, known in colloid chemistry as flocculation, finds an extensive practical use for sedimentation of colloid dispersions.

The size of the immune system cells is of the order of 10 μm, i.e., it lies in the size range of particles of typical organic and inorganic sols. The cellular surface formed by outer membranes is, generally tpeaking, a universal multisite heterofunctional sorbent for polyelecsrolytes and some other hydrophilic polymers. Figure 38 shows diagrammatically the cross section of a small area of a cellular membrane. It is formed by a double layer of lipids and built-in protein globules. Polar "heads" of lipids forming the outer surface of the double lipid layer contain anionogenic and cationogenic groups as well as groups capable of forming hydrogen bonds. Regions of polymer chains can also be sorbed on protruding membrane proteins that represent a substantial fraction of the total membrane surface. The familiar phenomena of agglutination of erythrocytes and platelets by polybases are, in physicochemical terms, very much like the phenomena of flocculation of conventional colloid particles by polyelectrolytes. Recall also findings on T-lymphocyte-to-macrophage "adhesion". The effectiveness of cellular "adhesion", like that of other colloid particles grows as the length of the polybasic macromolecules increases.

Now, supported by the available evidence, let us try to imagine

the fate of the linear polyelectrolyte added to the blood stream. Naturally, it first interacts with plasma proteins and blood cells "sticking" them together in various combinations and "sticking" different plasma proteins to the cellular surfaces. Physiological consequences of this interaction would be fortuitous and unpredictable if the randomly occurring contacts remained eventually unchanged. Yet this is not the case. The aforementioned possibilities of rapid reactions of macromolecular substitution (complex rearrangement) enable each macromolecule, wandering in the circulatory system in search of a local thermodynamic optimum, to change repeatedly, depending on the surroundings, its interaction partners or, at least, some of them. The polyelectrolyte administered to the organism ultimately collects in the liver, lungs and finally in the spleen during the period of its active cooperation with components of the blood and lymphoid system. Thus it contributes to the onset of a multitude of more or less lengthy intercellular, intermolecular (e.g., interprotein) and macromolecular-cellular (e.g., protein-cellular) contacts. In particular, it has been shown by *in vivo* assays that administration to the blood of salts of strong polybases, such as 2,5-ionen, immediately leads to a marked drop in the content in it of free platelets because of their agglutination by polycations. It will be recalled that 1 ml of blood contains approximately $10^{16}$ different protein molecules, some $5 \cdot 10^9$ erythrocytes also capable of sorbing polycations and as few as $5 \times \times 10^8$ platelets. The amount of polycations introduced in the blood in the aforementioned assays was about $10^{16}$ chains per ml. Therefore the very fact of agglutination by polycations of just platelets, amongst a host of competing partners must be considered as corroborating the assumption of lability of at least some of multisite cooperative contacts in which polyelectrolytes administered to the organism participate. Moreover, it suggests the possibility of a substitution of these contacts by others that are more advantageous in thermodynamic terms.

It is also usual to administer polymer immunostimulants in doses not exceeding the average concentration. This amounts to some $10^{16}$ chains per 1 ml of blood. The amount of T- and B-lymphocytes in 1 ml of blood is only some $3 \cdot 10^6$. However, proceeding from the above considerations, one may suppose that polyelectrolyte macromolecules do ultimately "reach" lymphocytes as well. They do so in part directly in the blood stream yet mainly as they further penetrate into the lymphoid tissues that contain most of T- and B-cells (some 99% of their total content in the organism).

*A hypothesis on the mechanism of polyelectrolytic immunostimulant activity.* Now we have ample evidence to propose a hypothesis on the mechanism of action of polymer stimulants.

It has been earlier pointed out that in most cases B-lymphocytes and T-helper cells are the imminent contributors to the immune re-

sponse to the antigen administered to the organism. Assume that at least one of the mechanisms of polymer-stimulated action is agglutination of B-lymphocytes with T-helper cells owing to multisite linear macromolecule adsorption on cellular membranes. This facilitates the necessary cellular cooperation. It seems likely that "adhesive" regions of the polymer embrace concurrently the antigen and other protein factors thus facilitating their localization at the B-lymphocyte surface where antigenic receptors are located. Viewed in the context of this hypothesis it is easy to understand the aforementioned structural and chemical diversity of polymer stimulants. The fact is

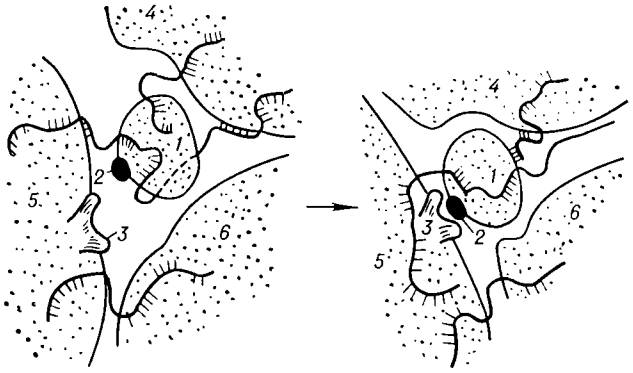



**Fig. 39.** Hypothetic scheme of self-assembly of immunologically active antigen-cell agglomerate involving polymer adjuvant:
*1*—antigen; *2*—hapten (antigenic determinant); *3*—receptor of B-lymphocyte; *4*—T-helper; *5*—B-lymphocyte; *6*—macrophage

that cellular membranes, as well as protein globules, are heterofunctional. Consequently, they represent sufficiently universal partners for cooperative sorption of a variety of polyelectrolytes. (It is possibly true not only for polyelectrolytes but also for other macromolecules showing a suitable hydrophobic-hydrophilic equilibrium.)

It would seem at this point that we have come up against a serious contradiction. The assumed mechanism of stimulation by polymer adjuvants of the intercellular and antigen-to-cell interaction might appear at first glance to be quite non-specific. It is common knowledge, however, that antigen-cell agglomerate needed to trigger the synthetic apparatus of the B-lymphocyte must be perfectly specific both in composition and mutual arrangement of its participants. The particular B-lymphocyte and the particular T-helper cell must correspond to each type of antigen. Moreover, a definite region of the antigen molecule must find on the B-lymphocyte surface the corresponding receptor and come to a structure-specific contact as diagram-

matically shown in the right-hand side of Fig. 39. Yet in fact the above contradiction is a seeming one. It will be removed if we recall that the aforementioned macromolecular substitution reactions permit an extensive search for all possible combinations of protein cellular components that enter into a multisite interaction with the polymer adjuvant. One is sure to find among the combinations tried-out ones that are suitable in terms of composition. Moreover, there is every ground to believe that the biological specificity of the immune system components, whose cooperation is responsible for antibody production against the particular antigen, is underlied by their maximum structural and chemical complementation. Then deeper energetic minima, compared with "incorrect" ones, should correspond to immunologically "correct" combinations of elements agglutinized by the polymer adjuvant. Should this be the case, then in the course of random rearrangements it is immunologically active combinations that would be gradually selected and fixed. To put it in other words, the macromolecule of a non-specific polymer stimulant operates much like a sorting device. Similarly to a seine, it first indiscriminately catches various cells and proteins (including antigens and immune cells) to form their agglomerates. Then this "device" changes parly one components for others until, by trial and error, combinations are found that are optimum in thermodynamic terms.

As to the correspondence of a thermodynamic optimum to optimum of immunologic activity, Nature has taken care beforehand. Therefore a non-specific polymer adjuvant facilitates, in the long run, self-assembly of highly specific antigen-cellular complexes. A randomly produced agglomerate (see the left-hand side of Fig. 39) undergoes rearrangements and, by trial and error, it finds a structural option diagrammatically shown in the right-hand side of Fig. 39. Thereupon the conventional mechanism of the immune response is triggered. The phenomena of a reversible structure-specific self-assembly in aqueous solutions have been rigorously proved by means of physicochemical methods for less complicated protein-synthetic polyelectrolyte complexes, in particular, BSA—PVP-$R_2$ systems. The latter appear in an electronic microphotograph as cylindrical particles. Each cylinder is made up of individual protein globules lined by polycations. As components react in a solution, random multisite contacts, naturally, first occur between them. This is, however, succeeded by very rapid recombination and formation of ordered cylindrical particles. What is essential is that in the excess of polycations the protein globules, first randomly distributed over them, immediately leave some of them in order to "satiate" others and become component parts of ordered agglomerates that in the given case are more advantageous in the thermodynamic framework. There is every ground to believe that such phenomena occur in more complex systems as well. In fact, we deal with the realization of the most general principle un-

derlying the self-assembly of a wide variety of cellular organelles, viruses and others where a structural thermodynamic optimum is also achieved to some measure by trial and error.

Much interest is evoked by recent evidence for the dependence of the level of the immune response on the length of the chain (i.e., the polymerization rate $n$) of polyelectrolytic immunostimulants.

The thermodynamic constant characterizing the strength of cooperative linkage of molecules is known to significantly (exponentially)

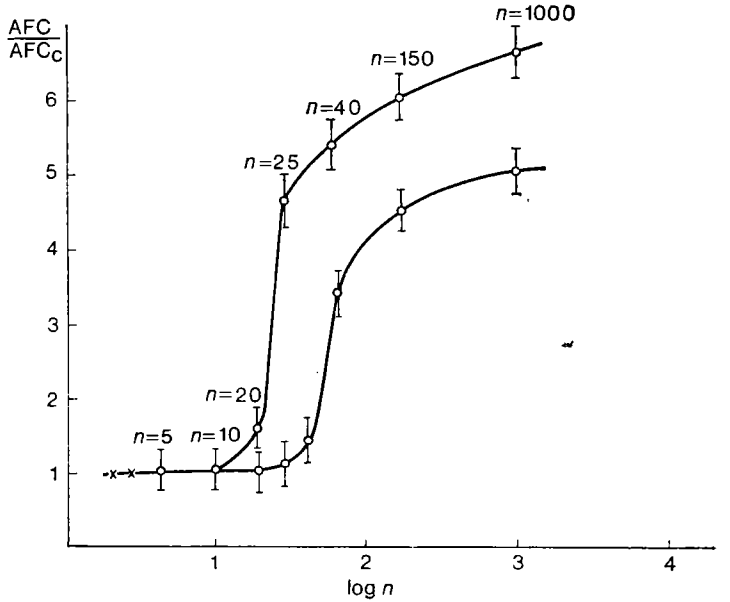



**Fig. 40.** Dependence of relative number of AFC in mouse spleens on degree of polymerization ($n$) of quarternized polyconidines:
*1*—PC-1; *2*—PC-2. Dose of polymer immunostimulants is 50 mg/kg. Immunization of mice with sheep erythrocytes is with dose $5 \cdot 10^8$. $AFC_c$ is the number of antibodies in control (without polymer immunostimulant)

depend on the polymerization rate. Correspondingly, the fraction of linked up segments, as $n$ increases, is drastically enhanced from 0 to a value approaching unity (i.e., 100%) over narrow interval of variation of $n$. Thus, the nature of the phenomenon itself appears to be critical. For this reason to establish the critical character of the dependence of the immunostimulant activity of a polyelectrolyte on the length of macromolecules is of crucial importance as we evaluate the validity of the hypothesis expounded above.

As objects of investigations we have chosen fractions of polycations PC-1 and PC-2 (for their structural formulae refer to previous sections). Figure 40 using semilogarithmic coordinates shows dependen-

cies of their immunostimulant activity on $n$ expressed as the ratio of the amount of AFC produced in assays involving a polyelectrolytic adjuvant to that of AFC in control. As can be seen from these findings, the adjuvant action is expressed only on attaining some critical values of $n$. Thereupon it dramatically increases to then practically attain the limit value. Thus experimental evidence is in full agreement with the assumption of a cooperative interaction of polyions with components of the immune system as being a key factor that determines their participation in the immune response.

It is noteworthy that PC-1 and PC-2 begin to "work" at different values of $n$. The presence in PC-2 of relatively more hydrophobic benzyl radicals seems to enhance the ability of its links for sorption on the surface of immune cells. Therefore "sticking" to membranes, other conditions being equal, occurs with a shorter length of the molecular chain. If one takes into account that membranes of B-, T-lymphocytes, macrophages and other cells differ in composition, it becomes evident that the sensitivity of the critical value of $n$ to the composition and structure of the link of a polyelectrolytic chain opens up, in principle, possibilities for "aiming" the polymer stimulant at cells of the required type. This is necessary to increase the selectivity of the action and to do away with side effects. Indeed, such a selectivity was detected, e.g., in the case of PAA. This latter exhibits properties of a mitogen and ionophore with respect to B-lymphocytes. In contrast, it produces no such effect on T-lymphocytes.

The dependencies of immunostimulant activity of PAA and PVP on $n$ with a fixed number of macromolecules administered to an animal are also of a critical character.

As has already been noted, the polyelectrolyte-induced enhancement of T-B-cooperation occurs only when the molecular chains attain definite lengths. This can be seen by looking at Fig. 41. It is of note that the dependence on $n$ of the ability of PVP-$R_2$ to "agglutinate" negatively charged particles of an aqueous polymer latex is of the same qualitative character. This can be detected from the increase in optical density (i.e., turbidity) of the system upon the addition of aqueous electrolytic solutions. Viewed in the context of our suggestions, these data are naturally explained in terms of a cooperative sorption of polycations on electrostatically complementary surfaces of the corresponding particles (whether T- or B-cells or latex particles) that results in their agglomeration.

We have already noted the structural and chemical diversity of polyelectrolytic adjuvants showing an approximately equal level of stimulating effect. This is, however, far from implying that the function of an immunostimulant can be performed by any high molecular weight polyelectrolyte. It follows from the above that the scope of this diversity is limited by the ability of polymer compounds to cement components of antigen-to-cell systems via the multisite in-

teraction. At the same time it is known from the physical chemistry of disperse systems that polyelectrolytes, adsorbing on the surface of disperse phase, can not only flocculate but also stabilize sols. Thus preventing agglutination of colloid particles (e.g., as a result of their recharging). The transition from polymer flocculants to stabilizers is governed in each particular case by a whole set of factors. Of these, an important role is played by the electrostatic equilibrium of the macromolecule. Therefore, generally speaking, no wonder that,

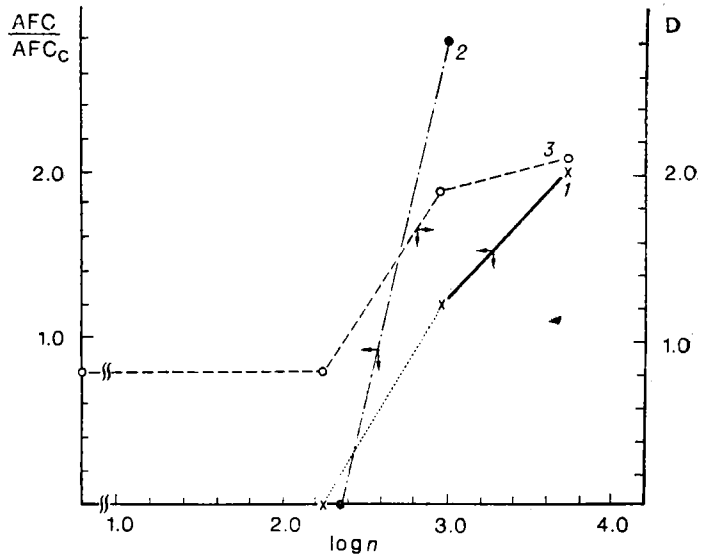



**Fig. 41.** Dependence of relative number of AFC generating as a result of administration of sheep erythrocytes in T- and B-cell culture on degree of polymerization of PVP-$R_2$ (*1*) and PAA (*2*).

Dotted line (*3*) corresponds to dependence of optical density (D) of butylacrylate-methylmethacrylate latex (63 : 67) with average particle size 0.15 μm on degree of polymerization of PVP-$R_2$ (weight concentrations of latex and PVP-$R_2$ are $2.5 \cdot 10^{-3}$ % by weight)

unlike PMVP and PAA homopolymers their copolymers of intermediate compositions (1:1 and 3:1) not only fail to stimulate, but, on the contrary, strongly inhibit the immune response to the injection of sheep erythrocytes. They, as it were, prevent T-B-cell cooperation.

The reported data, naturally, cannot be regarded as a proof of the "mechanistic" hypothesis on agglutination of components of the immune system as being the principal cause of stimulating the production of AFC. Yet, should one wish so, they can be treated in the framework of this hypothesis. In any case they fit well into it.

We are led further along the lines expounded by us by the following experiments. It has been disclosed that synthetic polymer adjuvants not only enhance the cooperation of B-lymphocytes and T-

helper cells but also substitute (at least, in part) for T-helper function. It seems to follow from these experiments that in the presence of polymer immunostimulants no T-helpers are needed to trigger the immune system. The investigated polyelectrolytes stimulate a T-independent immune response to the injection of a typical T-dependent antigen. It should be recalled that one of the essential T-helper functions is to furnish the B-lymphocyte a second non-specific signal. Remaining within the framework of ideas as the predominant role of the multisite adsorption of polyelectrolytes on cell membranes, we must accept that this signal is in the given case furnished by the polyelectrolyte adsorbed on the B-lymphocyte surface. It is too early to judge upon a concrete mechanism of providing a second signal but it is, however, clear that regions of chains "sticked" to the membrane may give rise to an appreciable change in its activities and characteristics. They may change the membrane potential, permeability or induce a phase transition in the lipid layer, etc.

The assumption as to the non-specific activation of B-lymphocytes by linear polyelectrolytes adherent to them is in agreement with the findings obtained from *in vivo* and *in vitro* experiments. In particular, the *in vitro* experiments have demonstrated that PAA and PVP added to a culture of splenic cells (i.e., B-cells) of intact mice increase the rate of cell division by a factor of 2.5 to 3.5. This can be adjudged from the rate of the inclusion of $^{3}$H-thymidine in the composition of DNA. The phenomenon has been established by *in vivo* experiments of polyclonal activation of B-lymphocytes under the effect of PVP, PAA and CP-PAA·VPD. It consists in the non-selective induction of transformation of different clones of B-lymphocytes into antibody producers (AFC) respectively against a whole array of various antigens that have not been administered to the organism.

It is noteworthy that, as has been demonstrated by *in vitro* physicochemical measurements, at the moment the process of B-lymphocyte division is triggered by PAA and PVP quaternary salts the rate of the transport of ions and some other low molecular weight materials through the plasma membrane markedly enhances. Already in the first 1-2 hours after the action of the polyelectrolyte there occurs essential enhancement of transmembrane fluxes of calcium ions and of precursor molecules of nucleic acids, thymidine and uridine, along the gradient of their concentration. This effect, which, broadly speaking, can be identified with the change of membrane permeability, seems to be non-specific with respect to the nature of the transported substances. It is, rather, an indication of "facilitated" transmembrane transport for all or many types of small molecules. In this it differs from the action of conventional ionophores (i.e., substances selectively enhancing the ion transport) or carrier proteins that are as a rule responsible for the transport of compounds of one particular type.

It has been further shown that sorption of polyelectrolytes on cell

membranes not only affected the passive transport but also enhanced the active transport, i.e., the transport of ions through the membrane against the gradient of their concentration. The active transport is known to be carried out by means of special membrane enzymes, ATPases. It emerged that addition of PAA to a culture of B-lymphocytes already in 1-2 minutes enhanced the total activity of ATPases. At this, activated were ATPases that brought about the transport of sodium, potassium and calcium ions.

A schematic diagram of an antigen-cell complex formed with the participation of polymer adjuvant that secures a T-independent im-

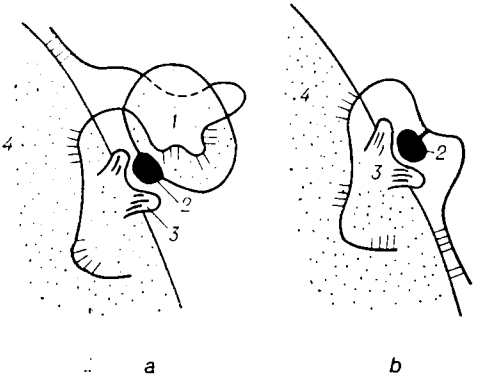



**Fig. 42.** Hypothetic schemes of antigen-cell systems inducing T-independent immune response:
*1*—antigen; *2*—hapten; *3*—receptor of B-lymphocyte; *4*—B-lymphocyte

mune response is presented in Fig. 42. It is believed that the antigen takes part in the formation of a structure-specific contact with the B-lymphocyte receptor (a first specific signal). A second signal is furnished by the B-lymphocyte surface polymer.

The described mechanism of eliciting a T-independent immune response in the presence of polymer adjuvants, like the mechanism of activation by the latter of T-B cooperation is, of course, also hypothetical. It is consistent with experimental findings and, consequently, can be used to account for them. However, strictly speaking, this is not sufficient to consider it as proved. To raise a hypothesis to the rank of a theory or a principle requires careful verification. The sections that follow deal with this question.

## Macromolecular Complexes as Superantigens

Thus, controlled-structure artificial polyelectrolytes strongly stimulate the immune response. They enhance T-B interaction, ensure the T-independence of the immune response to T-dependent

antigens, directly influence the B-lymphocytes, activating them, and stimulate a pronounced immune response in individuals of low-responder genotypes. This evidence is of great biological significance. We have already discussed the problem of genetic control of the immune response. Ir-genes realize their activity mainly through the synthesis of the T-lymphocytes that interact with the B-lymphocytes upon eliciting the immune response. Consequently, to induce the immunization effect in low-responder organisms to the given antigen (to convert weakly antigenic substances to strong antigens) it is necessary to seek pathways to a phenotypic correction. That is, we must find techniques to "bypass" the Ir-gene control of the immune response. Since the principal object of the Ir-gene expression is T-lymphocytes, one of the "bypasses" may be provided by the T-independence of the immune response to the particular antigen.

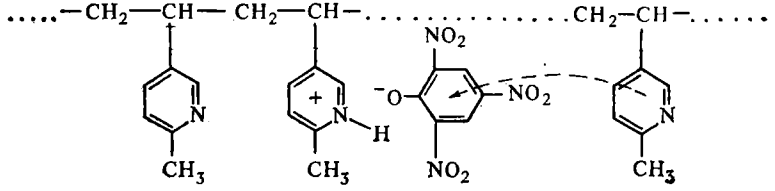
Apart from immunologic unresponsiveness determined by Ir-genes, congenital and acquired immunodeficient states are currently known. In these, defects of the immune system are localized at the level of the T-cell system. Consequently, use of polycations and polyanions as factors securing thymus-independence opens up the way both to a phenotypic correction of the immune response and to eliciting a T-deficient response under immunological deficiency. Naturally, the following idea was conceived. If a weak antigen is attached to a polymer macromolecule inducing the immune response and securing the T-independence, then such a system would ideally combine both antigenic specificity and stimulating activity even in the presence of low-response Ir-genes. Is it possibly a path leading to the development of "superantigens"?

**From polyelectrolytic immunostimulants to artificial antigens.** Indeed, the combination schematically presented in Fig. 42 resulted from random wanderings of its participants in the blood stream and lymphoid tissues. But why should we make antigen and immunostimulant look for each other? It is possible to rule out this stage by linking them. What new properties and expressions can be expected of a conjugate of a natural antigen and a "sticky" linear macromolecule using the proposed physicochemical scheme? In the first place, the level of conjugate-elicited immune response must be by far higher than the immune response to the same antigen induced by the same, but separated from it, adjuvant. In the second place, the higher immune response should be T-independent.

More than that. What if we attach the "sticky" macromolecule not to the antigen as such but to its "anchor" group, hapten? The latter is an antigen fragment which fails to induce the antibody production unaided but determines the specificity of antigenic interaction with the corresponding antibodies and receptors of the corresponding B-lymphocytes. In a contact, the antigen "recognizes" its B-lymphocyte because the hapten links to its complementary receptor at the cellu-

lar surface. However, the polymer carrier, as a result of multisite cooperative sorption at the B-cell surface may bring to the latter the hapten bound to it. Then, as a result of random wanderings of regions of a flexible chain along the membrane surface, the fragment of the hapter-carrying chain will then find the relevant receptor and adapt itself to it in such a manner that the hapten grouping will eventually be adequately fixed on the receptor in the right way. It is natural to suggest that it is due to the complementation of hapten and receptor that the minimum amount of the free energy of the entire system would correspond to such a state. Should the above considerations be correct, the hypothetical situation schematically presented in Fig. 42 would be sufficient for triggering a T-independent immune response. This is because a first specific signal would be furnished to the B-lymphocyte by the hapten, and a second, non-specific one, by components of the chain of the polymer carrier sorbed on the membrane. In other words, the hapten-polymer adjuvant conjugate must possess the properties of artificial thymus-independent antigen. This is what had to be first verified.

**First artificial antigen of a new type.** The studies were started with an attempt to develop an artificial antigen in which the role of a carrier would be played by PMVP molecule, and the role of an antigenic determinant by a simple chemical compound, a trinitrophenyl group:



Trinitrophenol, being a typical hapten, is unable to evoke the immune response (i.e., antibody production) until it is attached to a protein or other natural macromolecule. The attachment of the trinitrophenyl group to a polymer adjuvant led to complex exhibiting pronounced antigenic properties. It emerges that a trinitrophenol-PMVP complex induces a fairly high immune response consisting in the production of antibodies specifically reacting with the haptenic group. Antibodies to a protein-carried hapten are usually formed following an additional activation of the organism by special agents known as adjuvants. On the other hand, upon the immunization with a hapten linked to PMVP or PVP molecule, no such stimulation is required. Finally, as has turned out, the antibody production against artificial antigen trinitrophenol-PMVP is thymus-independent and needs no T-cells. T-deficient animals (B-mice) immunized with

a synthetic antigen generate as many antibodies or even more compared with normal individuals. Upon the immunization with the same hapten introduced into a protein molecule but not into a PVP molecule, T-deficient animals produce no antibodies.

**Serum protein-polyelectrolyte complexes as synthetic T-independent antigens.** Of great practical significance is the development of highly immunogenic artificial molecules bearing antigenic determinants of viruses, microbes and parasites, i.e., protein (polypeptides) and polysaccharide fragments. This is why in our further studies we make use of bovine serum albumin (BSA) as a model antigen. Being a very weak antigen, BSA usually evokes an immune response only upon repeated immunizations of the animals with an additional stimulation by adjuvants. The linking of BSA to a polyelectrolyte molecule was expected to induce a higher immune response to the protein determinant of this artificial antigen. Synthetic antigens were represented by a BSA-polycation complex, resulting from hydrophobic bonds between polymer chains and protein globules, or by a chemical conjugate in which BSA exhibits a covalent bond with the polycation. A copolymer was selected as a complex-forming polymer. At a neutral value of pH in aqueous solutions it is capable to produce complexes with BSA not only by forming electrostatic salt bonds but also by way of hydrophobic interactions of cetyl ($C_{16}H_{33}$) radical with non-polar regions of protein globules. For this reason in this particular case one could expect the formation of extremely stable protein-polyelectrolyte complexes not dissociating at the physiological values of ionic strength. In particles of the complex each polycation (1,000 links long) binds two protein molecules in aqueous solution.

Investigating a possibility of synthesizing highly immunogenic antigens, by linking specific determinants with stimulating polyelectrolyte carriers, it was also essential to evaluate whether it was effective to use covalent chemical bonds. Such an estimate is important both for the theory of the antigenic effect and in practical terms as viewed in the context of preparing compounds of a maximum stability. The presence in macromolecules of a copolymer of 4-vinyl pyridine and 4-vinyl cetylpyridinium bromide of free carboxyl groups permitted their chemical covalent bonding to BSA molecule amino groups via the activation stage with carbodiimide. The polycation polymerization rate was $10^3$. In this case one protein globule was attached to each copolymer chain.

The immunization of normal animals with the obtained synthetic antigens induced an immune response whose level was 50 to 100-fold higher compared with the immunization using pure BSA. Figure 43 demonstrates the curves of accumulation of AFC that synthesize antibodies to BSA in response to the administration of polymer-protein complexes and conjugates.

A very much pronounced immunogenic activity of the synthetic an-

tigen obtained by the attachment of protein molecules to a polyelectrolyte cannot be ascribed to the action of the polymer as a conventional immunostimulant. Upon a separate injection to animals of a protein antigen and a polyelectrolyte or upon an injection of unstable complexes disintegrating in the organism, an enhanced immune

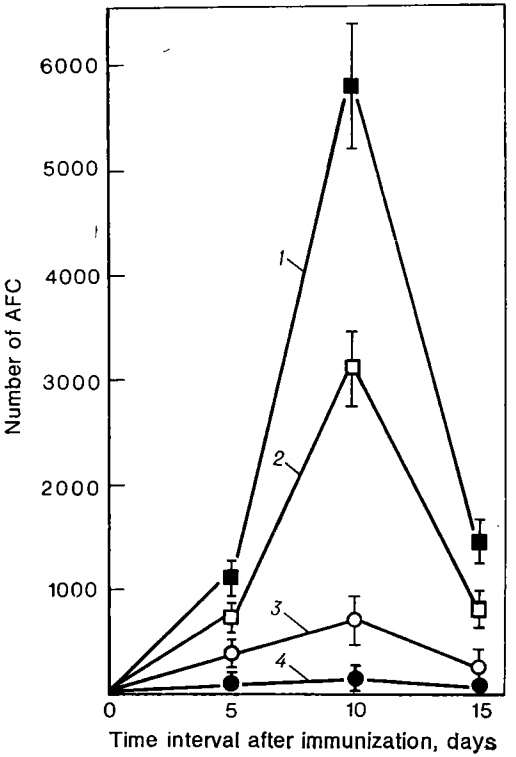



**Fig. 43.** Kinetics of accumulation in mouse spleens of AFC against BSA-determinants of synthetic antigens obtained by attaching BSA to copolymer on basis of PVP:

*1*—administration of covalent BSA and copolymer conjugate; *2*—administration of system stable at physiological values of ionic strength of BSA-copolymer conjugate stabilized with cetyl radicals; *3*—administration of electrostatic BSA-PVP-$R_8$ system relatively unstable at physiologic values of ionic strength; *4*—administration of pure BSA. (Doses in terms of BSA are in all cases identical)

response is also observed. However, this enhancement is incommensurate in magnitude with the immune response elicited upon the immunization with a protein-polyelectrolyte complex where antigen is attached by means of hydrophobic or covalent bonds.

The question as to the T-dependence of the obtained synthetic antigens remained open. To resolve it we conducted experiments in

nude mice, congenitally athymic animals lacking accordingly the entire T-lymphocyte system. It emerged that an injection of pure BSA (weak antigen) practically evoked no immune response in normal and athymic animals. The injection of BSA-containing antigen, as has been pointed out, strongly amplifies the immune response in normal mice. Yet even in athymic mice it likewise attained a fairly high level.

Finally, in the last series of experiments the aforementioned polycation carrier was linked to a relatively strong T-dependent natural antigen, bovine gamma-globulin (BGG). As was expected, the immunization of normal mice with such a complex evoked an immune response whose level exceeded by far control, i.e., the immune response of normal mice primed with pure BGG. The injection of BGG to athymic mice, naturally, brought about no antibody production since BGG is a typical T-dependent antigen. On the other hand, the administration of a BGG-polyelectrolyte complex elicited a powerful immune response in these mice as well.

Synthetized complex macromolecules can thus be considered as unusually potent artificial antigens. These are products made up of a protein and a synthetic polyelectrolyte. Each of the starting components taken separately is either not immunogenic (polycation) or weakly immunogenic (BSA). The prerequisite for the immunogenic action of polyelectrolytes linked to the molecule of antigen is the sufficient stability of this complex compound in the organism. The linking of both weak and potent T-dependent protein antigens to polymer adjuvants permits the production of potent T-independent artificial antigens that evoke the generation of antibodies to corresponding proteins.

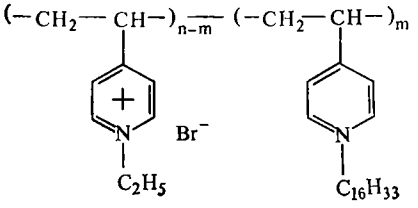
The next step was to obtain other polymer-protein conjugates using protein antigens of bacterial and viral origin.

**Polyvinyl pyridine copolymer complexed to tuberculin.** Thus, synthetic antigens have been developed by attaching non-natural polyelectrolytes to haptens or proteins. These antigens exhibit properties of T-independent antigens, elicit an enhanced production of antibodies without any additional adjuvants. Evidence, similar in principle, was obtained upon complexing polycations with microbial antigens.

It must be noted that the overwhelming majority of microbial antigens, including so-called "chemical" vaccines, are crude extracts of bacterial cells. It is quite evident that, in order to develop synthetic antigens, chemically pure homogeneous antigenic preparations are needed. They must be well enough characterized by their physicochemical properties. Otherwise it would be unfeasible to obtain stable preparations of synthetic antigens in which a polymer adjuvant is linked to the same molecules of antigen or its hapten fragments. One of fairly well purified and characterized microbial anti-

gens is PPD (purified protein derivative) of tuberculin. Tuberculin is a product of activity of tuberculosis mycobacteria excreted into the external habitat. Tuberculin is generally obtained from a nutrient medium in which mycobacteria are cultivated. Obtaining PPD requires careful purification of tuberculin.

PPD is a relatively weak antigen. Its injection to animals elicits the production of a small amount of AFC. PPD-polycation complexes of the following structure have been obtained:



As in the cases reported above of serum proteins (BSA, BGG), the attachment of the protein antigen of micobacteria to a polycation enhanced by far its immunogenicity. The immunization of animals with a PPD-polycation complex evoked a 40-50-fold immune response to PPD compared with the immunization using pure PPD. These experiments have provided the first evidence to support the above-formulated principle of developing synthetic antigens of a new type can be extended to protein antigens of microbial origin.

**Influenza virus hemagglutinin conjugated to polyelectrolyte.** Influenza virus hemagglutinin is the principal antigen eliciting the production of antibodies that neutralize the influenza virus. AFC generating in the organisms of individuals taken ill with influenza mainly synthesize antibodies to viral surface glycoproteid whose name comes from its capacity to agglutinate erythrocytes (hemagglutination).

To isolate hemagglutinin it was initially the practice to cultivate an influenza virus strain in 10-day old hen embryos since it proliferates well in their allantois cavity. Then M. Sinyakov (USSR), using specific, very sophisticated biochemical techniques, isolated hemagglutinin from a purified viral suspension. The preparation proved pure — in electrophoresis it was distributed as a single homogeneous fraction. Eventually A. Nekrasov (USSR) achieved its covalent bonding to a polymer immunostimulant (CP-AA·VP). The obtained synthetic antigen was used for mouse immunization. Incidentally, these animals, like humans, are liable to influenza. The immunization with a hemagglutinin-polyelectrolyte conjugate brought about more than a 40-fold increase in the production of AFC that synthesize antibodies to viral antigen.

It is a known fact, however, that antiviral antibodies produce a protective effect. They attack the virus circulating in the organism's fluids: blood and lymph. Should the virus penetrate into the cell, antibodies cannot "get hold" of it. In such a case T-killers come to their aid. They destroy the virus-infected cells thus killing both the virus carrier cell and the virus proper. Therefore the intent of the experiments that followed was to investigate the capacity of the artificial antigen to enhance cell-mediated immunity, i.e., the response elicited by T-cells. It appears that the synthesized conjugate enhances by an order of magnitude the cellular immune response, i.e., the T-killer activity.

The main result of this stage of our efforts is the validation of a possibility, in principle, to convert a weakly immunogenic viral antigen to a preparation of a high-level immunogenic activity. This can be achieved by its covalent bonding to a synthetic polymer carrier that performs the function of a biologically active adjuvant with a capacity to stimulate immunity.

**Alpha-fetoprotein and polyelectrolyte conjugate.** As is known, cancer antigens belong to weak ones. The organism is unable to develop an effective immune response against a forming tumour. It is not ruled out that the attachment of cancer antigens to immunostimulant polymer molecules will permit the preparation of anticancer vaccines. What makes this task appear as feasible is the fact that some cancer antigens have already been isolated in a pure form. Among these, in particular, is alpha-fetoprotein associated with primary liver carcinoma, and a carcinoembryonic antigen produced in the cases of intestinal, stomachal, esophagal and pancreatic adenocarcinomas.

The best studied of tumour antigens is alpha-fetoprotein. It is an embryonic protein whose synthesis discontinues in the post-natal period but may resume in some types of malignant degenerated cells. Like other cancer antigens, this protein, as such, is practically not immunogenic. That is, it elicits no immune response. Therefore the enhancement of alpha-fetoprotein immunogenicity is of crucial importance. This problem is of general significance since the reconstitution of synthesis of embryonic antigens in tumours appears to be a universal process in the biology of tumours.

It is customary to isolate alpha-fetoprotein from the blood serum of neonatant animals or from placental blood. The alpha-fetoprotein conjugated to the polyelectrolyte obtained in our laboratory elicited approximately 100-fold formation of specific antibodies compared with a pure antigen.

Thus by linking weakly immunogenic tumour-specific proteins to polymer adjuvants it is possible to develop artificial antigens that evoke a potent immune response against tumour antigens.

**(T, G)-A-L conjugated to polyelectrolyte as an Ir- and T-independent antigen.** One of likely applications of the method of developing

artificial antigens is related to the discovery of Ir-genes. It should be recalled that Ir-genes express their activity in close connection with the activity of the T-lymphocytes as well as in the processes of migration and cooperation of T- and B-cells. Based on these findings and results of the studies reported above, a general problem was raised with the intent to prepare synthetic antigens so as to avoid the Ir-gene control of the immune response. T-independent artificial antigens may provide a clue to the solution of the problem in hand. That is, they may open a pathway to a phenotypic correction of the gene control of the immune response. The reality of this promise is supported by the results of experiments in mice of different genetically pure strains using a synthetic (T, G)-A-L antigen by means of which an immune response gene was first discovered in mice, Ir-1-gene.

Completely synthetic antigens have been developed, conjugates of a synthetic (T, G)-A-L polypeptide with non-natural polyelectrolytes. In order to elicit the primary immune response to (T, G)-A-L, strain C57BL mice (bearing a high-responder Ir-1-gene) and strain CBA mice (bearing a low-responder Ir-1-gene) were immunized with pure (T, G)-A-L or conjugates of (T, G)-A-L with a copolymer of polyacrylic acid and N-vinyl pyrrolidone. To induce a secondary immune response (realization of immunological memory) the animals, previously immunized with (T, G)-A-L or conjugates of (T, G)-A-L with polyelectrolytes, were administered only (T, G)-A-L a month later. We have already mentioned that the action of the Ir-1-gene is expressed upon a secondary response. Yet there is another reason for studying this response. In fact, the expression of immunity against infection after a vaccination represents actually a secondary response, the expression of immunologic memory.

Following a repeated immunization of strain C57BL mice with solely (T, G)-A-L, much more AFC were produced than in CBA mice. This attributes to the Ir-gene function. The primary immunization with a (T, G)-A-L conjugated to polyelectrolyte evoked an enhanced immune response after a repeated injection of pure (T, G)-A-L to the organisms of mice of any strain used. What is more, not only does the number of developing AFC merely increase but also interstrain differences in antibody production are levelled off. In other words, what one observes is the phenomenon of a phenotypic correction of the Ir-1-gene-controlled immune response. Consequently, the immunization of animals with synthetic antigens, (T, G)-A-L-polyelectrolyte conjugates, elicits a powerful expression of immunologic memory despite the Ir-gene activity.

It emerged that the obtained conjugates were T-independent antigens. These induce an immune response in T-deficient animals.

Thus, a completely synthetic antigen, (T, G)-A-L conjugated to synthetic polyelectrolytes, is endowed with a potent T-independent immunogenic activity that elicits an Ir-1-independent immune re-

sponse. This ensures conversion of low responders to (T, G)-A-L to ones that are phenotypically high responders.

As is known, both T- and B-lymphocytes participate in the process of acquisition, storage and expression of immunologic memory. So, B-mice are incapable of developing immunologic memory to the T-dependent antigens. In B-mice "restored" with the T-cells immunologic memory is induced kept by both B- and T-lymphocytes. The

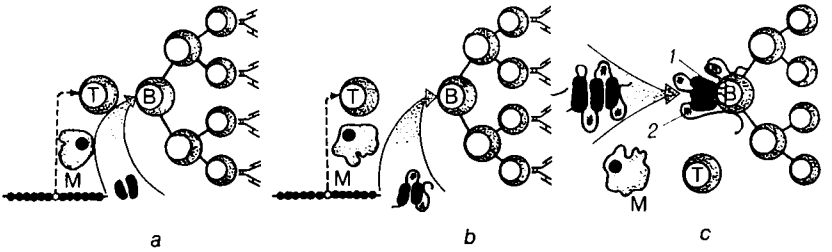



**Fig. 44.** Avoidance of genetic control over immune response:
*a*—native protein antigen is administered to organism: T-dependent immune response is triggered; *b*—synthetic antigen, protein and polyelectrolyte system are administered to organism: T-independent and Ir-independent immune response is triggered; *c*: *1*—information on antigen, *2*—signal for division. Circles represent Ir-genes. B—B-lymphocyte; T—T-lymphocyte

genetic defect of the Ir-gene is typically expressed at the level of T-helper cells that cooperate with B-cells in a T-dependent immune response. Synthetic polyelectrolytes ensure the T-independence of the immune response to T-dependent antigens. They seem to do so by the substitution for a second non-specific signal (see above) of T-helper cells. Hence one may assume that a phenotypic correction of Ir-gene control that occurs upon the immunization using an artificial antigen (T, G)-A-L polyelectrolyte may stem from a modification of the antigen by the polyelectrolyte that endows the former with the properties of a T-independent antigen.

Thus substituting for the function of T-cells, polyelectrolytes do enable one, as it were, to "bypass" the genetically predetermined immune response (Fig. 44).

## The First Artificial Vaccine

Thus, the first steps have been made toward the development of antigens of a new type. If antigens of some infectious agents against which immunity is not elicited or is weakly expressed raise an effective immune defense, then the principle of synthetizing vaccination molecules proposed by us will provide an approach to the development of a variety of artificial vaccines.

Synthetic polyelectrolytes we have employed have no natural analogs. These are not artificial replicas of protein molecules or their separate fragments. Nor is it possible to refer to the principle of their development as "mimicking nature". It is now difficult to predict what method of preparation of vaccination materials is likely to prove most promising, one of "mimicking nature" or one that involves a search for "non-natural molecules" that would have specified properties. What is clear is that such methods are sure to be found. Then it would be possible, indeed, to "assemble" antigenic determinants of various microbes and viruses on one polymer chain. This chain should ensure a powerful immune response to all antigens employed. According to our findings, the first experimental artificial vaccine may be believed to have been obtained.

**Conjugates of PAA with 0- and H-antigens of *Salmonellae*.** We have conducted experiments using a model of mouse typhus caused by *Salmonella typhimurium*, an infection typical of mice. *Salmonella* bacteria contain three antigens, 0, H, and K, principal of them being 0- and H-antigens. As noted, 0-antigen is a lipopolysaccharide contained in bacterial walls, H-antigen contained in flagellae of *Salmonellae* is a protein. Similarly to 0-antigen, K-antigen is localized in bacterial walls, however, it lies nearer to the surface and does not cover the entire bacterial cell.

The level of the immune response to the principal antigens of *Salmonellae* is known to correlate with the extent of immunity against this particular infection after reinfection (after immunization). It is, however, untenable to produce a sufficiently high protection effect upon the vaccination using these antigens or using a killed salmonellosis vaccine of genetically low-responder individuals to the given antigens. It has been demonstrated in our laboratory that an injection to genetically low-responder mice against 0- or H-antigens of PAA or PVP using these antigens induced a strongly pronounced immune response.

The most pronounced immunity to salmonellosis is elicited by priming the animals with PAA conjugated to 0-antigen polysaccharide and H-antigen. Recall that 0-antigen is a lipopolysaccharide complex whose lipid component is rather toxic. Therefore attempts were made to obtain an anti-salmonellosis vaccine from solely the polysaccharide, non-toxic component of the complex. Polysaccharide as such, however, elicits no significant immune response, being a hapten. That is why the attempts of using pure polysaccharide to elicit immunity to *Salmonellae* failed. As has been reported, we have succeeded in drastically enhancing the immunizing activity of polysaccharide isolated from 0-antigen through attaching it to a polyelectrolyte.

The next step in our endeavour was to investigate the protective properties of conjugates of *Salmonella* antigens with a polyelectro-

lyte. Mice were immunized using a conjugate of PAA with 0-antigen polysaccharide and H-antigen. After a definite period all animals (both immunized and non-immunized, control ones) were infected with *Salmonellae*, an absolutely lethal dose being administered. All control, non-immunized animals died in the first week after the infection. Individuals that had been previously immunized survived. The antigens proper (polysaccharide and monomer of H-antigen), in practical terms, did not protect the animals against death.

Thus, a first artificial vaccine of a new type providing an effective defense against mouse typhus has actually been prepared.

**Preparation of non-toxic polymer immunostimulants.** What is the degree of toxicity of polymer carriers and what ultimately occurs to them in the organism? It should be noted at this point that the employment of the aforementioned "sticky" polyelectrolytes and of artificial antigens developed using these latter will present no problem for the immunization of animals. Moreover, the problem of vaccination of humans with synthetic antigens appears to us to be likely to be resolved. The fact that one would not have to meet rigorous structural chemical requirements to polymer carriers in order to attain the principal immunologic effect permits an extensive search aimed at the lessening of side effects. It is not ruled out that detailed information of the composition and structure of immunoactive cellular membranes coupled to experimental studies of the interaction of potential polymer carriers with immunoactive cells in the presence of other competitors would permit the synthesis of more specific carriers. Such carriers, as they seek immunologically "adequate" partners, would be involved in fewer side reactions.

As to the synthesis and selection of such adequate polymer carriers that would be capable of being decomposed in a definite period and be fully expelled from the organism, such a task belongs to the domain of chemistry of polymers. As such, it is unlikely to present any principal difficulties. One of practically feasible pathways is this. Now that, thanks to the use of synthetic polymer carriers of most diversified structures, the general principle of development and functioning of T-independent immunogenic conjugates has been formulated, it is possible, guided by this principle, to employ as "sticky" polyelectrolytic components basic or acidic biodegrading natural or synthetic polymers. It is them that can be combined with the predetermined antigenic determinants.

Examples demonstrating, in principle, the possibility to develop non-toxic immunostimulant polyelectrolytes can be provided, in particular, by the already reported copolymers of acrylic acid and N-vinyl pyrrolidone (CP-AA·VPD). In a definite composition range these compounds are practically non-toxic though preserve immunostimulant properties. We have selected VPD as a toxicity-lowering component. Its homopolymers are known to find extensive use in

clinical practice as a blood substitute. At the same time polyvinyl pyrrolidone (PVPD) is endowed with a pronounced complex-forming activity and is water-soluble. We believed that an addition of VPD links to the PAA chain would permit lowering the polyelectrolyte toxicity and concurrently preserving an immunostimulant activity. Stemming from this assumption we synthesized a few copolymers of various compositions. All of these are fairly well soluble in water at neutral values of pH. As expected, their toxicity decreases with the decrease in their content of acrylic acid links. $LD_{50}$ for copolymers CP-AA·VPD-6 and CP-AA·VPD-5 amounts, respectively, to 800 and 625 mg/kg, i.e., they are practically non-toxic substances.

The administration of these copolymers to B-mice immunized with sheep erythrocytes brought about an appreciable enhancement of the immune response. In other words, these copolymers, similarly to PAA or PVPD, have the capacity to substitute for the T-cell helper function. Consequently, they may provide components for the synthesis of artificial antigenic conjugates according to the above proposed principle.

There is reason to believe that a controlled "dilution" of cationogenic or anionogenic links in macromolecules is a general approach of searching for the necessary range between the levels of acute toxicity and sufficiently expressed immunostimulant activity.

**Conjugates of 0 and H-antigens coupled to non-toxic polymer carriers—adjuvants.** These are actually polymers that are similar in structure to polymer blood substitutes that have found extensive use in medicine. It is hoped that non-toxic polymer adjuvants will be shortly employed for practical purposes. One of their major likely applications has to do with the principle of developing artificial vaccines of a new type we have elaborated.

To verify this assumption we have again employed *Salmonella* antigens. A polysaccharide and a monomer form of flagella H-antigen were chemically attached to copolymer CP-AA·PVD. Mice were immunized with antigens thus produced. Two weeks later the animals were infected with an absolutely lethal dose of virulent *Salmonella typhimurium*. As in the experiment reported above, the control (non-immune) animals died 5-7 days following the infection. A conjugate of polysaccharide and flagella antigen with copolymer gave all animals a protection against disease and death. The findings of this and a variety of other experiments have demonstrated that *Salmonella* antigens conjugated to a polyelectrolyte provided the defense of animals against death caused by *Salmonella typhimurium* 40-50 times as effectively as the initial forms of these antigens.

Artificial antigens and vaccinating preparations of a new type are likely to be applied in most diverse fields of immunology and in medicine generally as well as in veterinary science. One of the crucial tasks is prophylaxis of yet unconquered infectious diseases of man

and domestic animals. The synthesis of immunostimulant compounds free of the toxic effect opens up new pathways toward activation of immunity with a view to correcting primary (genetically determined) and secondary immunodeficiencies responsible for acute and chronic infectious complications in surgical, therapeutical, obstetric, pediatric, and other clinics. What underlies this task is to obtain and apply stimulators of particular links of the immune system or artificial compounds that substitute for lymphocyte humoral factor activities. Artificial antigens may also be employed as a remedy to cure allergies. The fact is that this impaired immune function is due to the synthesis of IgE-antibodies in the organism. This is why to combat allergies it is essential to find methods for switching the synthesis of allergic IgE-antibodies to the synthesis of antibodies of other classes endowed with protective functions. This problem may be resolved by means of polymer carriers for allergy-inducing substances so as to ensure such synthesis switching.

* * *

Thus, the attachment of antigens and antigenic determinants to non-immunogenic high molecular weight* polyelectrolytic immunostimulants is the general principle of and the general pathway toward artificial immunogens and, presumably, artificial vaccines. It ensures a higher level of the immune response, its T-independence and avoidance of the Ir-gene control. Undoubtedly, the mechanism of effect of macromolecular immunostimulants that are part of artificial immunogens of this type is intimately connected with their ability for cooperative sorption at immunocompetent cell surfaces, i.e, their membrane activity. Differently speaking, the membrane activity of the polyelectrolyte carrier ultimately expressed by its mitogeneity is a prerequisite for the occurrence of the set of the enumerated immunologic properties of a conjugate as a whole. The effectiveness of combining the antigenic and mitogenic polyelectrolytic entities in one complex macromolecule has been shown by the many reported examples. The recent attempt by M. Sela to develop an artificial vaccinating preparation by conjugating a synthetic polypeptide antigen to low molecular weight mitogens like muramyldipeptide, broadly speaking, is closely related to the strategy of our own approach.

The search and recognition of the proper clone of immune cells by the conjugate seems to be conducted by trial and error. It is carried out by conjugate migration from one partner to another via the

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* The lower limit of the "high molecular weight" is in the given case determined for each particular polymer by the aforementioned critical character of the dependence of its immunostimulant activity of the polymerization rate.

mechanism of cooperative macromolecular substitution and exchange as was rigorously proved and studied in detail on model systems. We can imagine a polyelectrolytic chain "wandering", together with the attached antigenic entity, up to a certain time about its protein-cellular surroundings and palpating, as it were, the surfaces of particles encountered. This notion rests upon a solid physicochemical foundation and provides the necessary stage in the suggested concept. The ultimate goal of the search is the energy minimum corresponding to the formation of an immunologically competent protein-cellular agglomerate.

The brilliant idea to develop vaccinating molecules previously suggested by M. Sela implies the synthesis of a set of unique specific antigenic determinants identical to natural ones followed by the construction from these of macromolecules concurrently carrying several such fragments. Similar macromolecules are expected to represent synthetic analogs of a polyvalent vaccine and to immunize against several infections at a time. It should be emphasized that the method suggested by M. Sela and one proposed by the present authors are in no way competitive. On the contrary, they add to each other. Indeed, Sela's "nature-like" vaccinating macromolecules are in principle likely to be coupled to polyelectrolytic stimulants. This may be done, for instance, to enhance the level of the immune response or to provide for its phenotypical correction. The studies reported above give convincing evidence of such an eventuality. On the other hand, high molecular weight non-immunogenic polyelectrolyte immunostimulants may be used to attach to them a set of Sela's specific antigenic determinants.

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*Printed in the Union of Soviet Socialist Republics*